SYMBIOTIC NITROGEN FIXATION AND SEED DEVELOPMENT OF
GENETICALLY MODIFIED SOYBEAN IN RELATION TO
BRADYRHIZOBIUM INOCULATION AND NITROGEN USE UNDER
ACIDIC AND SALINE DYKELAND SOIL CONDITIONS

by

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Submitted in partial fulfilment of the requirements
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DALHOUSIE UNIVERSITY

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The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "SYMBIOTIC NITROGEN FIXATION AND SEED DEVELOPMENT OF GENETICALLY MODIFIED SOYBEAN IN RELATION TO *BRADYRHIZOBIUM* INOCULATION AND NITROGEN USE UNDER ACIDIC AND SALINE DYKELAND SOIL CONDITIONS" by Panchali Katulanda in partial fulfilment of the requirements for the degree of Master of Science.

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ABSTRACT

The objectives of this study were to examine the effects of starter N inputs and *Bradyrhizobium* inoculation on soybean symbiotic N fixation and grain yield under field and greenhouse conditions. The study was conducted in the Wellington and the Habitant dykelands in NS. The treatments consisted of 0, 1.5, 3 and 4.5 g/kg seed rates of inoculant and 0, 10, 20, and 30 kg/ha rates of N fertilizer. Under acidic soil conditions, the inoculated plants showed significant N fixation responses in the Wellington field while saline soil conditions suppressed N fixation in the Habitant field. The soybean grain yield showed an increasing trend with the inoculant rate 4.5 g/kg seed. The starter N fertilizer did not facilitate the soybean grain yield in the dykelands. Under controlled environment conditions, inoculant rate 3 g/kg seed alone produced the same amount of yield as 1.5 and 4.5 g/kg seed rates with N fertilizer.

LIST OF ABBREVIATIONS AND SYMBOLS USED

A	Photosynthesis rate
BNF	Biological Nitrogen Fixation
BTB	Bromothymol Blue
Ci	intercellular CO_2 concentration
CRD	Completely Randomized Design
DAP	Days After Planting
E	Transpiration rate
EC	Electrical Conductivity
Gs	Stomatal conductance
HI	Harvest Index
N	Nitrogen
NHI	Nitrogen Harvest Index
NN	Nodule Number
NS	Nova Scotia
NSAC	Nova Scotia Agricultural College
PDW	Plant Dry Weight
PFW	Plant Fresh Weight
PGA	Peptone Glucose Agar
PN	Pod Number
PODW	Pod Dry Weight
POFW	Pod Fresh Weight
RBM	Residual Biomass
RBMN	Residual Biomass Nitrogen
RCBD	Randomized Completely Block Design
RDW	Root Dry Weight
RFW	Root Fresh Weight
RU%	Relative Ureide Percentage
SW	Seed Weight
TBM	Total Biomass
TBMN	Total Biomass Nitrogen
YMA	Yeast Manitol Agar

ACKNOWLEDGMENTS

CHAPTER 1   INTRODUCTION

1.1 INTRODUCTION

Dykeland developed from marshlands, is a unique agricultural resource found in Atlantic Canada. Nova Scotia has over 75% (or 18,000 ha) of the total Atlantic Canada's dykeland resources (Anonymous, 1987). Most of the dykeland soils have poor internal drainage and excess salts, which limit the crop growth (Rodd et al., 1993). Historically, dykelands have been used for hay production.

At present, soybean is grown as animal feed for dairy production in dykelands. Soybean is one of the highest value crops grown in dykeland. Since, soybean is rich in protein and oil, proper management of growing conditions is necessary to harvest its full potential.

Growing high quality of soybean is needed to ensure high quality dairy production. Soybean contains 42% of protein and 19.5% of oil (Wilcox and Shibles, 2001). Therefore, the crop is a heavy user of nitrogen (N). Soybean N requirements are met by either soil mineral N acquisition or symbiotic N fixation. To obtain the maximum yield of soybean, it is necessary to use N fixation by root nodules and absorb N from soil (Harper, 1974). It has been reported that high nodulation and high N fixation rates increase soybean yield (Burias and Planchon., 1990). Furthermore, the inoculation of soybean with *Bradyrhizobium* species increased the seed protein content (Egamberdiyeva et al., 2004).

However, the ability of soybean to fix atmospheric N is not always adequate for yield maximization (Wesley et al., 1998). Several soil and climatic factors have an effect on N fixation in soybean under field conditions. Studies revealed that soybean grain yield

is enhanced through N fertilizer application (Gan et al., 2002; Barker and Sawyer, 2005; Taylor et al., 2005; Osborne and Ridell, 2006; Tahir et al., 2009). However, crop production is not constantly increased with N rates. Excessive N fertilizer appears to be subjected to loss from the root zone and pollute the ground water (Li et al., 2003). The balanced supplement of the amount of N nutrients required by the particular crop is necessary to increase yields (Li et al., 2006).

There is research-based information for N fixation in soybean for non dykeland soil. Inoculation of soybean with *Bradyrhizobium* strains improves the plant dry matter, N concentration, N accumulation, and grain yield (Diaz et al., 2009). High rates of N fertilizer reduce the number of nodules and the nodule dry weights of the plant (Taylor et al., 2005). The application of 200 kg ha^{-1} of N fertilizer without inoculant does not improve the soybean yield compared with inoculated plants in loam and sandy loam soils (Aldbareda et al., 2009). Thus, there is a lack of research-based information about soybean and N nutrition relations under the specific dykeland conditions to produce high quality soybean grains. In this research, the soybean symbiotic N fixation and the grain yield responses to *Bradyrhizobium* inoculant and the fertilizer N application in dykelands were studied.

1.2 LITERATURE REVIEW

1.2.1 Dykelands

1.2.1.1 History of Dykelands

The dykelands were built up with silt and clay that was carried by the spring tide from the Bay of Fundy. Over thousands of years, tides deposited sediments (layers of silt) along the riverbanks to a depth of more than 40 cm. After salts leached out, the lands became productive for crop growth. The early Acadian settlers who had come from the lowland regions of France found huge, muddy, flat, salt marshes along the coast. They found the reclamation of the dykelands through ditching and dyking was easier than clearing the forest (Anonymous, 1987).

Preventing inland water movements into the dykelands and the discharging the fresh water accumulated in sources behind the dykes into the sea were the two major challenges associated with the reclamation of dykelands. Early settlers used "aboiteau", a wooden tunnel covered with marsh mud and sods with an inside hinged door to build the dykes. Since, 1948 the government built large concrete and steel "aboiteaux" to keep the upstream lands tides off (Anonymous, 1987).

Early Acadian settlers utilized the salt marsh grasses as a fodder for livestock and later they started to plant European grasses such as timothy (Ganong, 1903). They discovered that these lands could produce abundant crops year after year without adding fertilizers. Today, dykelands cover 18,000 and 15,000 ha of lands in Nova Scotia and New Brunswick, respectively (Bishop et al., 1968). Approximately, 33,000 hectares of

dykelands in Maritime province produce hay, corn, soybean and root vegetables; while others produce grass meal as a food for hogs and cattle (Anonymous, 1987).

1.2.1.2 Characteristics of Dykeland Soils

The topography of dykelands is level to undulating (Bishop et al., 1968). Dykeland soils were classified as Acadia soil or Gleyed Regosols which formed by the tidal action. These soils are believed to be derived from soft Carboniferous, Triassic sandstones, and shale from the surrounding uplands and those underlying the Bay of Fundy (Brydon and Heystek, 1958). Although, the soils are pedogenetically young, they are one of the first soils used by European settlers +for agriculture in North America. The soils have silty clay loam texture with fairly uniform particle size distribution. The soil profiles have a weakly expressed horizon development (A-B-C horizon sequence) with moderate to strong structural development. These soils contain a Bm horizon, where cation exchangeable sites dominate with magnesium (Mg^{2+}) ions (Beke, 1990). Dykeland soils containing high levels of salts have sodic or saline characteristics (Rodd et al., 1993).

1.2.2 Morphology and Development of Soybean Plant

The soybean is a dicotyledonous plant, which has an epigeal emergence (Koda et al., 1988). This leguminous plant is native to East Asia. Soybean plant shows an erect, sparsely branched, and bush-type growth habit with pinnately trifoliate leaves (Wilcox, 1987). The unifoliolate node produces the first true leaf, which is an un-trifoliate (Koda et al., 1988).

The vegetative stages of the soybean plants are determined by considering the number of nodes on the main stem starting with the unifoliolate node, which produces a first completely unrolled leaf (only two leaflets). The unifoliolate node is the first node where the plant produces its first true leaves. These leaflets are fully developed and located immediately opposite on the main stem. For example, if the plant contains five nodes, it is in V5 stage (Figure 1.1) and the plant with 18 nodes belongs to the V18 stage (Fehr et al., 1971).

Identification of the reproductive stages varies depending on the plant growth habit. In indeterminate type cultivars, start flowering, when about half of the nodes on the main stem develop. In these plants, flowering occurs continuously upward as plants produce new nodes. The flowering, the pod formation, and the seed development are predominant in the bottom portion of main stem. In the determinate type cultivars, flowering starts after all nodes on the main stem are developed. Therefore, the flowers, the pods, and the seeds are equally distributed all over the plant. Fehr et al. (1971) identified the developmental stages, which can be applied to all soybean genotypes grown in any environment (Figure 1.1).

At V 1 stage the unifoliated node, produce a completely unrolled leaf. V2 stage, first node above the unifoliolated node produces a completely unrolled leaf. There will be three nodes on the mains stem including with the unifoliolate node at V3. In the beginning of the reproductive stage (R1), there is one flower in every node. At R2 stage there is a flower immediately after the topmost completely unrolled leaf. At R3 stage, the length of the pod of four uppermost nodes with completely unrolled leaf is 0.5 cm while at R4 stage 2 cm.

Vegetative Stage

| Germination | V$_1$ | V$_2$ | V$_5$ | V$_6$ |

Reproductive Stage

| R$_1$ | R$_2$ | R$_3$ | R$_4$ |

| R$_5$ | R$_6$ | R$_7$ | R$_8$ |
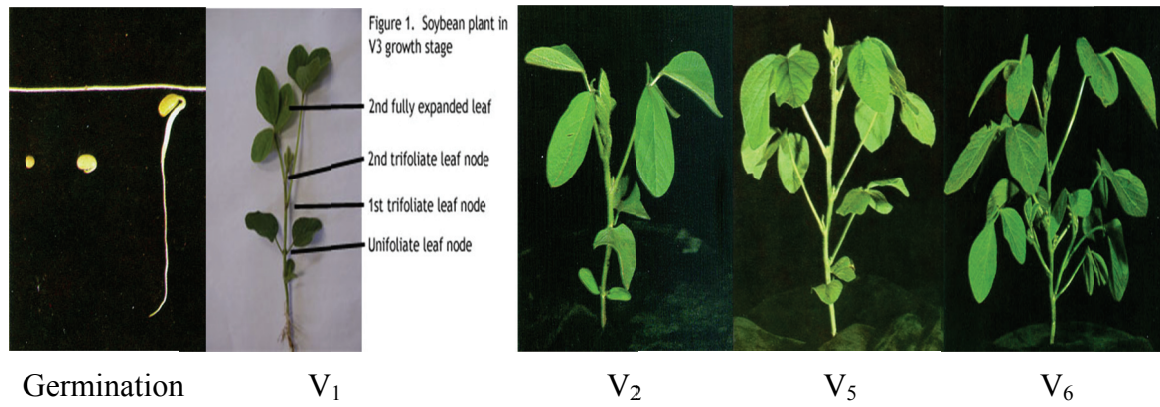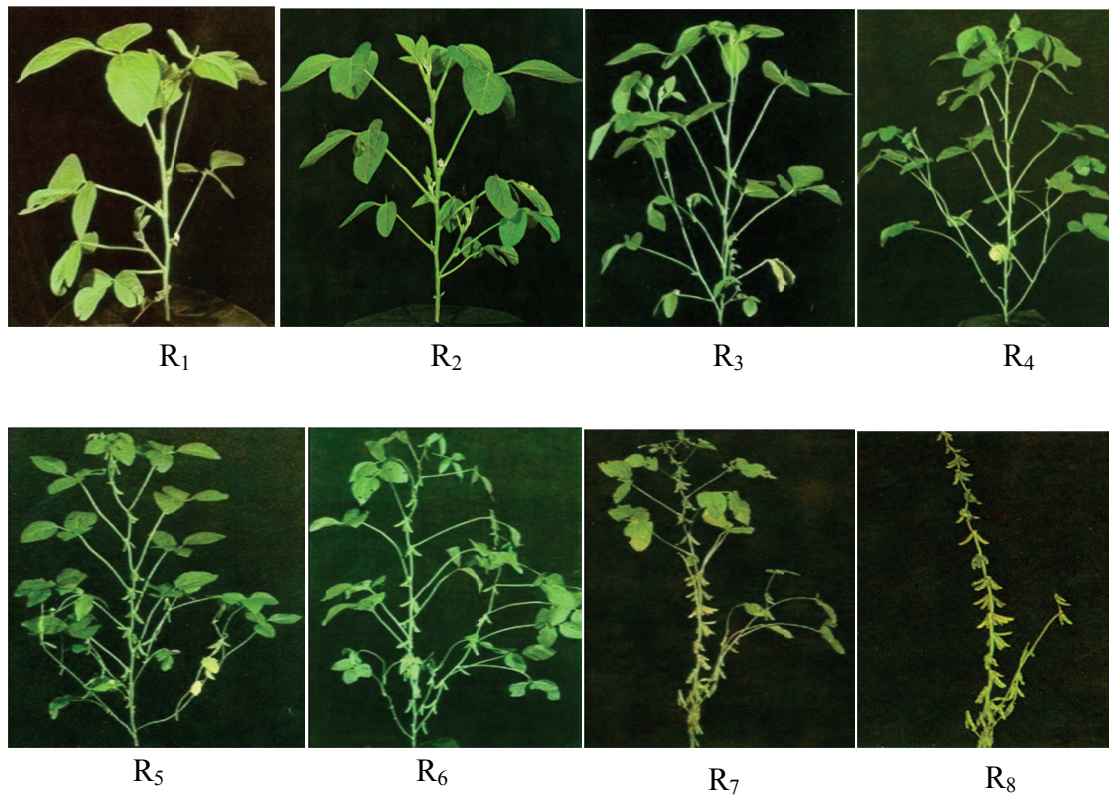
Figure 1.1 Stages of development descriptions for soybean plants

Adopted from www.ag.ndsu.edu

At R5 stage beans started to develop at one of the four uppermost nodes with completely unrolled leaf. The full size green bean can be obtained at one of the uppermost node at R6. R7 is the physiological maturity stage while R8 is the harvest maturity stage (Fehr et al., 1971).

1.2.3 N Nutrition and Plant Relations

1.2.3.1 N Cycling Processes in Plant and Soil Systems

Nitrogen cycling occurs in the atmosphere, the biosphere and the pedosphere. N exists in both inorganic and organic forms as well as many different oxidation states. In soil, about 95% to 98% of total N is bound to organic complexes, while the rest of the inorganic N is readily available to the plants. Whereas, availability of N is low in soils containing high levels of fixed ammonia (NH_4^+) (Stevenson, 1982). The predominant form of soil N is nitrate (NO_3^-) and it is suceptible to leaching. Further, soil N is bioavailable as NH_4^+, which is usually bound to the soil particles through cation exchange, and reduces the leaching losses. Organic N acts as a form of slow releasing nurient source for the plants. The release of soluble N from organic compounds largely depend on the charcteristics of the decomposer environment. The inorganic N released through mineralization and nitrification is dissolved in soil suspension (Heathwaite et al., 1996).

1.2.3.2 N Nutrition and Plant N Uptake

The N consumption of plants varies from one plant species to another. Within the species, the N amount varies depending on the genotype and the enviorenmental factors. There is a considerable variation among the plant parts (grain, stem, root, leaves etc.)

interms of relative amounts of N content. In general, most of the N is stored in the harvesting parts (seeds in most grain crops) than in stover, vines, stem, roots or straw. N aquisition can vary depending on the soil N status, agronomic practices and the climate (Stevenson and Cole, 1999).

Plants generally take up nutrients from the soil solution through the root system. The soil mineral uptake by plants becomes effective process due to the larger surface area of roots and their ability to absorb ions at low concentrations (Taiz and Zeiger, 2006). The two N fractions that can be utilized by the plants are inorganic N (NO_3^- and NH_4^+) and decomposable organic N (Schulten and Schnitzer, 1998). Usually NO_3^- concentration in the soil solution is low. Ammonium (NH_4^+) ions are tightly bound to cation exchange sites and present in low concentrations in soil solution. In aerated soil, NO_3^- is the predominant form of plant available N. First, the NO_3^- is absorbed into the root cell wall space and then transported to the plant cell. Nitrate uptake is occurred by either active or passive transportation depending on the NO_3^- concentration in the soil solution (Novoa and Loomis, 1981).

The soybean plant has protein-rich seeds and requires high levels of N to attain greater yield (Sinclair and DeWitt, 1975). It is reported that there is a good correlation between the total amount of N accumulated by the plant and the seed yield (Tewari et al., 2004). At the vegetative stage, plants are capable of absorbing mineral soil N rapidly and the leaf tissue has high NO_3^- content. As the plant reaches the reproductive stage (flowering), there is a rapid reduction in tissue NO_3^- content. There is a gradual decline of tissue NO_3^- content from flowering to early pod filling stage (Thibodeau and Jaworski, 1975). The maximum N fixation (acetylene reduction) is observed from late flowering to

early pod filling stage (Marcus-Wyner and Rains, 1983). At pod filling stage, developing ovules act as a competing sink for photosynthate resulting in a rapid decline in N fixation at mid pod filling stage (Thibodeau and Jaworski, 1975).

Nitrate uptake in the soybean can occur either in light or dark conditions. During the seedling and early vegetative stages, the plant N uptake is saturated at very low level of soil NO_3^- concentrations (0.5 mM) (Wilcox, 1987). The maximum NO_3^- uptake of soybean plant can be observed during early to mid-pod filling stages. The increase in the plant NO_3^- content with plant age is resulting from the increase in root mass rather than the increase in specific rate of NO_3^- uptake. The assimilated NO_3^- is temporary stored in the soybean roots or translocated to the shoots where NO_3^- reduction occurs (Wilcox, 1987). Nelson et al (1984) reported that 60% of seasonal N_2 fixation in soybean occurred after R5 stage and there was a high correlation between the seasonal acetylene reduction and the soybean yield. Symbiotic N_2 fixation is considered the main source for soybean seed protein synthesis.

1.2.4 N Nutrition of Plant

1.2.4.1 N Compounds and Metabolism in Plants

Ammonium taken up by the plant can directly enter into the amino acid synthesis pathway. In addition, absorbed NO_3^- has to be reduced to NH_4^+ before entering into the amino acid synthesis pathway (Novoa and Loomis, 1981). The reduction of NO_3^- is occurred in the cytosol by the NO_3^- reduction enzyme. The produced NO_2^- enters the chloroplast of the shoot and is reduced to NH_4^+ by nitrite reductase enzyme. The NH_4^+ assimilates to the amino acid pathway, which serves as the substrate for the transamination reaction to produce all the amino acids and proteins (Tischner, 2000).

N is a constituent of many important molecules, such as proteins, nucleic acids, certain hormones (eg. cytokinin, indol-3- acetic acid) and chlorophyll (Hopkins and Huner, 2004). Most of the N absorbed by the plants is translocated to the leaves through the transpiration stream and lesser amount of absorbed N is assimilated to amino acids in those organs. The amino acid synthesis is mostly occurred in the leaves, whereas the root exports very little amount of amino acids (Novoa and Loomis, 1981).

1.2.4.2 Soybean Responses to N Fertilizer Application

Application of starter N at an early vegetative growth stage or flowering can increase the pod yield and crop biomass by 44% and 16%, respectively. The proportion of the plant N derived from the N fixation is highest when N is applied at the pod filing stage where the plant N demand is high (Yinbo et al., 1997). It has been reported that the use of urea $((NH_2)_2 CO)$ or ammonium nitrate (NH_4NO_3) as the starter N fertilizer at rates of 8, 16, and 24 kg ha^{-1} promoted the early plant biomass and plant N compared to the no N treatment. Further, the soybean grain yield increased by 16 % at the N rate of 16 kg ha^{-1} over control treatment, with no improvement either in seed protein or oil content (Osborne and Riedell, 2006).

Schmitt et al (2001) conducted a study to identify the effects of application time, application method, and the source of N on soybean plant growth, grain yield, protein, and oil content at 12 sites. The study concluded that in-season application of N fertilizer did not increase the soybean grain yield or the oil content. However, there was a combine effect of all above factors on increasing soybean protein content at a rate of 0.4 g kg^{-1} (Schmitt et al., 2001). The soybean grain yield, protein, oil and fiber content did not

increase with the fertilizer N rates of 45 and 90 kg ha^{-1} (urea/slow releasing N) application at early reproductive stage (Barker and Sawyer, 2005). The early application (V2/R1) of N as a top dressing at a rate of 25 kg ha^{-1} promoted the soybean plant total biomass and the N accumulation during the seed filling stage (R5) which boosted the grain yield (Gan et al., 2003). N top dressing application at the seed filling stage (R3/R5) could not improve the plant total biomass, N accumulation and the grain yield (Welch et al., 1973; Gan et al., 2003). N top dressing application at R1 and R3 stages drastically reduced the soybean nodulation, whereas at V1 stage there was an optimistic effect, which increased the soybean nodulation (Gan et al., 2003).

The broadcasting of fertilizer N as urea (50 and 100 N kg ha^{-1}) at the pod formation (R3) and the seed filling stage (R5) increased the available N at the top 30 cm of soil compared to the unfertilized plots. However, increase in soil NO_3^- availability during the seed filling stage had no relevant effect on leaf senescence and the seed growth (Gutiérrez-Boem et al., 2004). The response of the soybean towards the fertilizer N was not temporally stable (Lambert et al., 2006).

1.2.5 Biological N Fixation

Atmospheric nitrogen (N_2) makes up about 78% of the air in the atmosphere; it is a colourless, odourless, tasteless and chemically an inert gas at the room temperature. This huge reservoir of N_2 is not available for organisms. In order to be utilized by the plants and the animals, the inert N_2 must be broken down to reactive compounds that can be easily metabolised. Further, N atoms must be bonded chemically with oxygen and

hydrogen through the N_2 fixation process and carbon through N assimilation process (Vitousek et al., 2002).

Earth's atmosphere contains 78% (4×10^{21} g N) of N_2 gas; however the plant and the animals do not have easy access to utilize the atmospheric N_2 for their growth. This is mainly due to the stability of the N_2 molecule. The triple bond between the N atoms requires large amount of energy to break. Annually 3×10^{14} g of N is fixed as NH_4^+ (Rees et al., 2005). The amounts of energy required to break the triple, double and the single bonds of N_2 molecule are 225, 100, and 39 Kcal mol^{-1}, respectively (Howard and Rees, 1994). Before assimilation, N_2 must be fixed and converted into the biologically usable forms. The most common forms of fixed N_2 are NH_4^+ and NO_3^-. In biogeochemical N cycle, the N_2 fixation is the process of converting atmospheric N_2 into NH_4^+ (Fisher and Newton, 2002).

Reduction of N_2 into NH_4^+ requires high amount of activation energy. To produce NH_3 by Haber-Bosch reaction needs the temperature of 300-500 ^{o}C and the pressure over 300 atmospheres in the presence of Fe based catalysts. In the nature, the limited group of organisms called as diazotrophs are capable of converting atmospheric N_2 into metabolically usable forms of NH_4^+. These diazotrophs can range from free-living forms to associations with various plants (Kim and Rees, 1994).

Leguminous plants are able to fix atmospheric N_2 through the association with *Rhizobia*. *Rhizobium* is a bacterium, which is hosted by the root system of certain legume plants. The legume plant supplies the carbohydrate for bacterial growth while the bacteria fix atmospheric N_2 into NH_4^+, to be converted into plant usable amino acids (Russelle, 2008). Symbiotic association is a highly specified relationship between the host plant and

the bacteria. Rhizobium-legume symbiosis involves the interaction between the plant and the bacteria leading to initiation and development of the root nodules (Trichine, 2006). N fixing symbiotic association is a mutualistic interaction between the plants that belongs to the family leguminosae and the soil bacteria genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Rhizobium* (Broughton et al., 2000). These organisms live in nodules as N fixing bacteroids. A single *Rhizobial* cell that infects a root hair can increase the progeny by 10^{10} within few weeks. These organisms adapted to different types of environmental conditions since the genes are not necessary at the free-living stage and these genes become "turned on" only after interacting with the host plant (Russelle, 2008).

1.2.5.1 Biochemistry of Biological N Fixation

Atmospheric N_2 fixation is catalyzed by the nitrogenase enzyme. The prokaryotes having gene coding for nitrogenase enzyme are capable to fix atmospheric N_2. The nitrogenase enzyme present in bacteroids consists of two metalloproteins designated as iron (Fe) protein and molybdenum-iron (Mo-Fe) protein which catalyze the energy dependent reduction of N_2 (Hopkins and Hürner, 2004). The Mo-Fe protein contains the active site for the substrate reduction. There are three steps involved in dinitrogen reduction (Figure 1.2) (Rees et al., 2005). First, the Fe-protein is reduced by electron carriers such as flavodoxin and ferredoxin. Then, single electron is transferred from Fe-protein to Mo-Fe protein in Mg-ATP dependent process. Finally, the electron is transferred to the substrate, which already bound to the active site of Mo-Fe protein complex and the cycle is repeated until sufficient electrons and protons form to reduce the substrate (Kim and Rees, 1994; Rees et al., 2005).

$$8 \text{ H}^+ + 8 \text{ e}^- + \text{N}_2 + 16 \text{ ATP} \longrightarrow 2 \text{ NH}_3 + \text{H}_2 + 16 \text{ ADP} + 16 \text{ P}_i$$
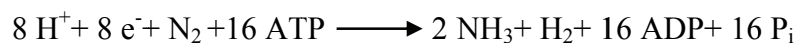
Biological N fixation (BNF) is an energy dependent process. If the free-living N_2 fixing bacteria are non-photosynthetic and they require chemical energy, while photosynthesis diazotrophs utilize light energy. Microorganisms, living in the rhizosphere or associating with the root obtain energy materials from the plant. The *Rhizobia* legume symbiosis can provide large amount of N to the plants than free-living organisms (Saikia and Jain, 2007).
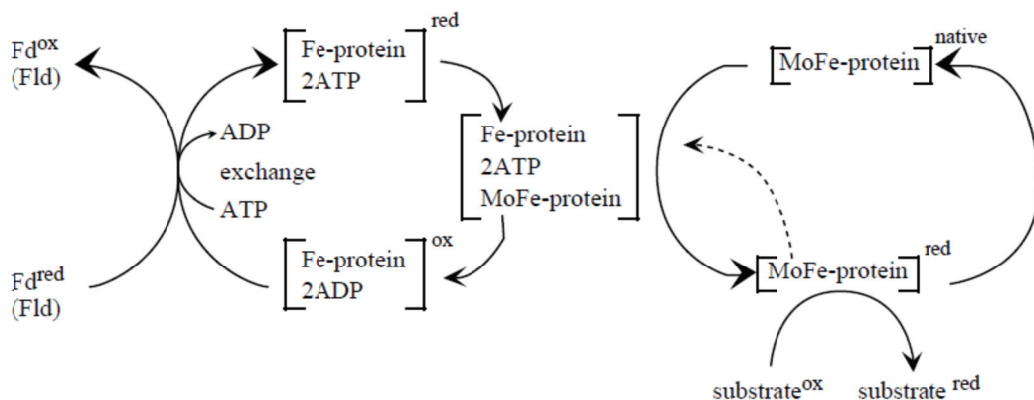
Figure 1.2 Schematic diagram of N turnover cycle, illustrating the flow of electrons from electron carriers to the Fe protein and Mo-Fe protein and subsequent reduction of substrate (Adopted from Rees et al., 2005).

1.2.5.2 Mechanism of Nodule Formation

The nodule establishment occurs due to the sequence of multiple interactions between the bacteria and the leguminous plant (Hopkins and Hürner, 2004). The root nodule development can be divided into three main stages as pre infection, nodule initiation, and differentiation. There are three different signal types, exchange between root and bacteria in the nodule formation. Legume seed coat contains different types of flavonoids in large quantities. These flavonoids act as chemo-attractant for the

corresponding root nodule forming bacteria and induce *rhizobium* nod gene (Mylona et al., 1995). With the presence of flavonoids of host plant, *Rhizobia* colonized and multiplied in the rhizosphere. The colonized bacteria in the rhizosphere begin to synthesis nod factor, which are derivatives of "chitin". Nod factor induce morphological changes (increased root hair production and development of shorter thicker roots) in the host root (Hopkins and Hürner, 2004). In response to chemical signals, *Rhizobia* attach to the newly (viable) immerged root hairs in two steps (Figure 1.3). The *rhzobia* first loosely attach to the root hairs by using a bacterial surface protein. Later, they attached to the root hairs tightly by using cellulose fibrils (Hirsch, 1992). The lectins on root surface and complex polysaccharides of symbiont are the two major unique recognition molecules involving in rhizobia-host interactions (Hopkins and Hürner, 2004).
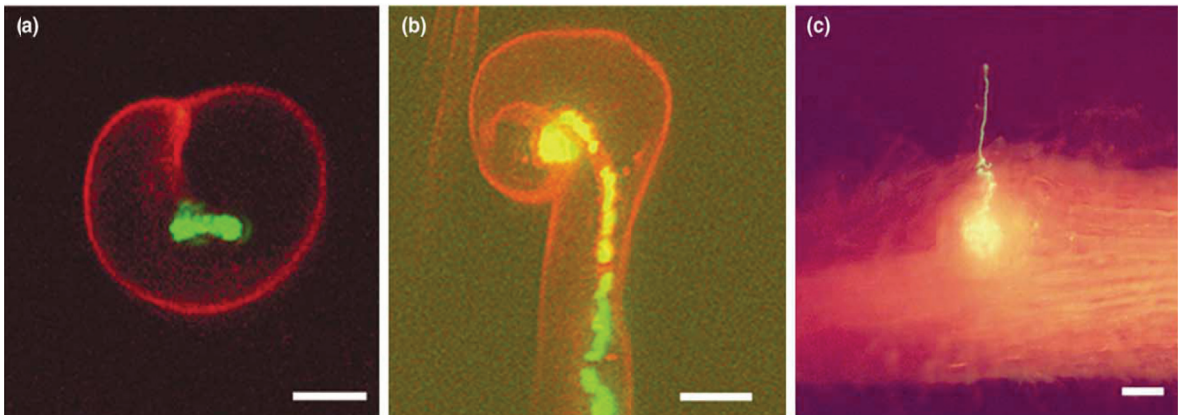
Figure 1.3 *Rhizobial* infection process. (a) Induce of root hair formation by Rhizobia. (b) Infection thread growing inside the curled root hair. (c) Infection thread growing towards the primodia formed by inner cortex cells (Adopted from Geurts et al., 2005).

The root nodule development is induced by lipochitin-oligosaccharide signals secreted by the bacterial microsymbiont. Infection occurs via infection threads that pass through the root hairs into the cortex and release bacteroids (Karas et al., 2005). In the presence of node factor producing *Rhizobia,* root hairs get curled as a result of

depolarization of the plasma membrane, change in the flux of calcium (Ca^{2+}), proton efflux, rearrangement of the active filaments and increase cytoplasmic streaming (Hirsch, 1992). Curling occurs due the continuous redirecting of the growth towards the one side of the root hair where the bacteria attach. The root hairs sense the node factor secreted by the bacteria and grow towards it, turning 360^{0} and entrapping the bacteria in the pocket formed by the curl. The *Rhizobia* degrade the cell wall and the plasma membrane and enter into the plant cell where they trigger the growth of infection threads, which penetrate into the root cortex (Figure 1.3). In the host cells, bacteria differentiate into bacteroid, which is surrounded by a peribacteroid membrane (Hopkins and Hürner, 2004). The nodule primodia formation is initiated within the root cortex (Geurts et al., 2005).

1.2.5.3 Different Types of Root Nodules

Rhizobium, *Bradyrhizobium* and *Azorhizobium* are the three genera associated with legume plants. After pre-infection of the roots cortical cells division takes place. Type of nodule form depends on the place of cortical cell division, which is determined by the host plant. Cell divisions occurs either in inner or outer cortex of the root. There are two types of nodules as determinate and indeterminate (Figure 1.4). Indeterminate type nodules consist of persistent nodule meristem, while determinate type lacks persistent meristem. Meristematic region is a zone of the nodule where cells actively divide and differentiate. Because of the continuous cell division, the indeterminate type nodule structure elongate resulting in a club shaped nodule. The plants, having indeterminate type nodules are alfalfa, clover, and pea. Determinate nodules are spherical in shape. The cell division occurs at the beginning, whereas only the cell enlargement is

facilitated during the later growth resulting in a spherical shape. Nodules of soybean, common bean, and mung bean are examples for the determinate type nodules (Hirsch, 1992).
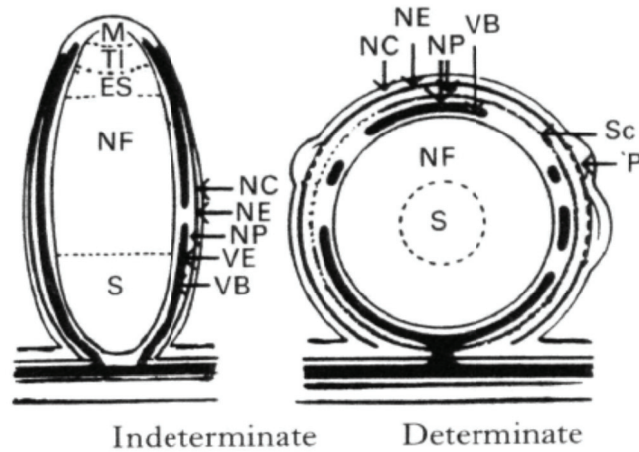
Figure 1.4 Schematic diagrams of indeterminate and determinate type nodules (Adopted from Hirsch, 1992).

The origin of the indeterminate type nodule is the temperate region while the determinate type is tropical or subtropical. Some of the characteristics of the indeterminate type nodules are the initial cell division in inner cortex, production of a broad infection threads and transportation of fixed N_2 as amides. In determinate type nodules, initial cell division occurs in the outer cortex of the cell. Also, they produce narrow infection threads and fixed N_2 is transported as ureides (Hirsch, 1992).

1.2.5.4 N Transport between the Nodule and the Plant

As mentioned earlier, the root nodule functions as source of N and sink of carbon source of the legume plants. Sucrose is transported as carbon source from leaves to the nodules. This is degraded by sucrose synthase in the nodule, and introduced to the nodule's metabolic pathways. Ammonium is exported from the microsymbiont as the

first product of the N fixation in all indeterminate and determinate type nodules. The NH_4^+ is assimilated to the cytoplasm of the nodule cells to the glutamine synthase pathway. Depending on the nodule type, glutamate is transformed into different N transport forms (Mylona et al., 1995).

In determinate type nodules, glutamate is converted into ureides. In the nodules, the glutamine synthase is present in both infected and uninfected cells of soybean. In uninfected cells, uricase catalyzes the conversion of uric acids into allantoin. Allantoinase catalyzes the next step of purine oxidase in uninfected cells. In determinate type nodules, the uninfected cells also participate in N transportation, where ureide is transported to the uninfected cell through plasmodestmata. In indeterminate type nodules, no specific function has been assigned for the uninfected cells. As a result, an efficient transport of fixed N_2 is achieved (Mylona et al., 1995).

Fixed N must diffuse as NH_4^+ across the peribacteroid membrane. Within the plant cytoplasm, the NH_4^+ is assimilated by glutamine synthatase to glutamine. Glutamine is converted to glutamate by transferring the amide group to α- ketoglutarate and catalyzed by glutamate synthase. Although the glutamine is the principle organic product of N fixation, in the legumes of tropical origin (eg. soybean and cowpea), ureide is the predominant form, translocating the fixed N. In synthesis of ureide, allatonin and allantoic acids are formed by the oxidation of purine nucleotides (Hopkins and Huner, 2004).

1.2.5.5 N Fixation Responses in Soybean

Soybean is capable of fixing large quantities of atmospheric N_2 resulting significantly high yield (Imsande, 1989). It has been reported that the proportion of N derived from BNF of the plant total N was 50% in soybean grown in a soil having a moderate N content (Hardarson et al., 1984). In a soil of low N content, the soybean plant can fix 300 kg of N ha^{-1} in the presence of effective *Rhizobial* strains. Soybean forms N fixing symbiosis with either *Bradyrhizobium japonicum* or *Sinorhizobium* species (Keyser and Li, 1992). Seed inoculation with *rhizobium* can increase the total N and grain yield in early maturing soybean cultivars. The total N accumulation and N fixation are low during the early growth stage and then they increase rapidly at later stage (Sanginga et al., 1997).

N fixation reaches maximum at R3/R4 stage, and then drops (Sanginga et al., 1997). During the seed filling stage, the translocation of fixed N is greater compared to N derived from the soil (Koutroubas et al., 1998). Rapid N fixation during the grain filling stage enhances the net photosynthesis rate and respiration leading to higher amount of usable N in soybean plant. N fixation is energy dependent process. *Rhizobium* generates energy required for N fixation through oxidation of host plant photosynthates. At R5 stage, high demand for photosynthates from pods and nodules facilitate the initial rate of energyzation of the thylakoid membrane and stimulate the photosynthesis (Mury et al., 1993). The higher photosynthesis rate at R5 stage increased the plant biomass and the soybean grain yield (Imsande, 1989). Rapid N fixation during the pod filling stage increased the seed yield and protein content (Imsande, 1992). It has been reported that the

soybean residual N contribution to the soil was approximately 18 kg N ha^{-1} (Sanginga et al., 1997).

1.2.5.6 Factors Affecting BNF

There are several environmental factors affecting BNF. The process of N fixation is strongly related to the physiological states of the host plant. The severe environmental conditions such as salinity, unfavourable soil pH, nutrient deficiency, mineral toxicity, extreme temperature conditions, low or extremely high levels of soil moisture, inadequate photosynthates, and disease conditions can affect the plant growth and development. As a result, the persistent *rhizobium* strains will not be able to perform root infection and N fixation in their full capacity (Zahran, 1999).

The rate of BNF is highly variable and depends on bacterial strain, legume cultivar, soil, and environmental conditions (Shantharam and Mattoo, 1997). The moisture stress can adversely affect the nodule functions. The drought conditions can reduce nodule weight and nitrogenase activity. After exposing to the moisture stress for 10 days, the nodule cell wall starts to degrade resulting in senescence of bacteroids (Ramos et al., 2003). Under salinity conditions, the accumulation of Na$^+$ reduces the plant growth, nodule formation, and symbiotic N fixation capacity (Soussi et al., 1998; Kouas et al., 2010). High salt level can directly affect the early interaction between the *rhizobium* legumes in nodule formation (Singleton and Bohlool, 1984). The plant nitrogenase activity reduces dramatically as a result of formation of ineffective nodules at high temperature (40 $^{\circ}$C) (Hungria and Franco, 1993).

Extreme soil pH can reduce the *Rhizobial* colonization in the legume rhizosphere. N fixation can be inhibited by low soil pH (van Jaarsveld, 2002). The characters of highly acidic soils (pH < 4) are low level of phosphorous, calcium and molybdenum along with aluminum and manganese toxicity, which affects both plant and the *Rhizobia*. As a result, of low soil pH conditions nodulation and N fixation is severely affected than the plant growth. Highly alkaline (pH > 8) soils tend to be high in sodium (Na^+), chloride (Cl^-), bicarbonate (HCO_3^-) and borate (BO_3^-) which reduces the N fixation (Bordeleau and Prévost, 1994). Uddin et al. (2008) revealed that the nodule number (NN) and size were significantly inhibited by the application of N fertilizer (urea). Symbiotic N fixation varies according to the carbon allocation to the nodules, in relation to endogenous factors, current photosynthesis, crop growth rate and other competing sinks for carbon (Voisin et al ., 2003).

1.2.6 Quantification of Symbiotic N Fixation

There are different methods to quantify the symbiotic N fixation. Some of these methods are non-destructive while other methods are destructive. Depending on the method of quantification, estimates of BNF can vary. One of the commonly use method is total N difference method. In the method amount of N fixed is quantify by using plant total N of N fixing plant and non-fixing reference plant (Hardarson and Danso, 1993). Acetylene reduction assay is one of the methods that adopted to measure the N fixation in legume plants. The principle of this method is that the N_2 reducing enzyme can reduce the acetylene (C_2H_2) to ethylene (C_2H_4). In this method, whole plant or plant part is incubated in a closed system, which contains 10% C_2H_2 gas for 0.5-2 hours. The C_2H_2 and C_2H_4 are measured by using the flame ionization gas chromatography (Minchin et al.,

1994). This method often shows a good qualitative agreement with direct measurement of symbiotic N fixation.

In ^{15}N dilution method, an estimate of different N sources in the plant is measured. Prior to planting, a small quantity of ^{15}N enriched fertilizer is incorporated to the soil. The proportion of N obtained by the plant through BNF is calculated by assuming the non-fixing reference crop uptake is similar to the ratio of soil mineral-N and fertilizer ^{15}N of the N fixing plant (McAuliffe et al., 1958). The drawbacks associated with this method are high fertilizer cost, decline of plant available ^{15}N in the soil over the time, and non-uniform distribution of ^{15}N along the soil depth (Witty, 1983).

Natural abundance method is one of the methods extensively used to quantify the N fixation. Soils are enriched with ^{15}N in relation to the atmospheric N_2. N fixing plants will have a ^{15}N abundance intermediate between the atmosphere and the reference plant relies on the soil mineral N, which reflects the proportion of N_2 fixation and N uptake. The amount of N gain through N fixation is obtained by multiplying the proportion of N fixation by the total plant N (Myrold et al., 1999). The natural abundance is expressed as parts per thousand deviations from the atmospheric N_2. This method facilitates the measurement of N fixation without disturbing to the system when both fixing and non-fixing reference plants are present (Peoples et al., 2002).

Herridge and Peoples (1990) described about the use of ureide method to determine the symbiotic N fixation of the field grown soybean. They explained the ability to use vacuum extracted stem sap and stem extract methods to calculate the N fixation. The estimate of N fixation by ^{15}N ranged between 68% and 59% for ureide are highly

correlated (r^2 = 0.97). The proportion of the N derived from the N fixation is not varying depending on the plant genotype and strain of *Rhizobia* (Peoples and Herridge, 1990). Further, Herridge and Peoples (1990) reported that the relative abundance of ureide -N in root bleeding sap, vacuum extracted sap ([100× ureide-N]/ [ureide-N+ α-amino-N+ Nitrate-N]) and stem extracts ([100× ureide-N]/[ureide-N+ Nitrate-N]) are highly correlated with the proportion of plant N derived through N fixation. The stem ureide method is effectively used to quantify N fixation under dry field conditions (Elowad et al., 1987).

1.3 OBJECTIVES

The objectives of my research were as follows:

1) To evaluate the individual and interactive effects of seed inoculation and varying rates of starter N inputs on soybean symbiotic N fixation, plant growth and yield in dykeland soils.

2) To isolate and characterize the *rhizobium* associated with dykeland soils.

CHAPTER 2   ISOLATION OF *RHIZOBIUM* FROM DYKELAND SOILS

2.1. INTRODUCTION

Soybean is one of the important field crops grown in North America as a feed for the dairy industry. Soybean nodulating *Rhizobia* are genetically diverse and classified into different genera and species. Based on the phylogenetic and phenotypic characteristics, *Rhizobia* are categorized into five different genera; *Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium* and *Sinorhizobium* (Young, 1996).

The response of soybean to inoculant is inversely related to the indigenous *Rhizobial* population in the soil (Thies et al., 1991). If the indigenous *Rhizobial* population is over a previously define threshold level, benefits of inoculation may not be obtained (Singleton and Tavares, 1986; Moawad et al., 1988). Failure of the inoculant can occur due to the greater number of competitive *Rhizobial* strains in the soil (Moawad et al., 1988). Legume inoculation with native *Rhizobia* has been shown to enhance plant growth (Rodríguez-Echeverría and Perez-Fernàndez, 2005). The existing *Rhizobia* in dykeland soils of Nova Scotia could have wider adaptability to the particular soil conditions. Inoculating with a *Rhizobia* which has a wider adaptability may improve soybean symbiotic N fixation on dykelands.

2.2 OBJECTIVES

The objective of this study was to isolate and characterize the *Rhizobium* associated with soybean root nodulation in the Habitant and the Wellington dykelands.

2.3 MATERIALS AND METHODS

2.3.1 Capture Experiment

A trap host study method was used to isolate the *Rhizobium* from the dykeland soils. The capture experiment was performed on April 2010 in a growth chamber at the Department of Plant and Animal Sciences of the Nova Scotia Agricultural College (NSAC), Truro, NS. Soybean cultivar "Lynx RR" was used as the trap host.

Prior to the capture experiment, the growth chamber and the pots were surface sterilized with a bleach solution and later wiped with 75% of ethanol (C_2H_5OH) to avoid contamination. Sterilized Pro-mix BX *(Premier Horticulture, Canada)* was used as the growing medium. The medium was moistened by adding water and placed in a shallow autoclavable tray, covered with an aluminum foil and sterilized in the autoclave for 15 minutes at 121 ^{o}C. After the sterilization, the Pro-mix was kept in a clean environment covered with the aluminum foil and then transferred into 2 L pots.

Soybean seeds were placed in a previously sterilized Erlenmeyer flask covered by a bottom half of a sterilized Petri dish. Then, the seeds were rinsed with 95% C_2H_5OH for 10 minutes and drained. Further, the seeds were swirled with 3% hydrogen peroxide (H_2O_2) for 3-5 minutes. The sterilized seeds were rinsed 4-5 times with sterilized distilled water. Then, the seeds were submerged in sterilized distilled water and kept in a refrigerator for 4 hours (Somasegaran and Hoben, 1985). Finally, the seeds were sown (10 seeds/pot) in the growing medium using sterilized forceps.

One week after germination, the seedlings were thinned out leaving three healthy plants in each pot. The seedlings were treated with different volumes of soil suspension

prepared from the dykeland soils. For this, fresh soil samples (10 samples) were taken at a depth of 0-20 cm from randomly selected locations in the Habitant and the Wellington fields. The fields were subjected to a crop rotation of corn-soybean-grass-soybean. The soil type is an Acadia marine loam (silty clay loam in texture), whose soil classification is referred to in Appendix A. Ten grams of representative soil sample were mixed with 100 ml of sterilized distilled water (10^{-1} dilution) and shaken on the rotary shaker *(Model KS, 130 CS 1, IKA, KS, USA)* for 2 minutes (Freidericks et al., 1990; Coutinho et al., 1999). The pots (three seedlings) were inoculated with four rates of 10^{-1} soil suspension. This was carried out to reduce the *Rhizobial* population density and facilitate the nodule formation with different *Rhizobial* strains. They were; 0 ml (S0), 1 ml (S1), 2 ml (S2) and 3 ml (S3). As the control treatment (S0), 1 ml of sterilized distilled water was added. Each treatment consisted of three pots (6 replicates). In addition to the plants grown in the Pro-mix, sterilized soybean seeds were also directly sown in pots containing Wellington and the Habitant dykeland soils. The pots were arranged in a completely randomized design (CRD) in the growth chamber.

The growth chamber conditions were; day and night temperatures of 25/20 ^{o}C, and relative humidity of 80% (Gan et al., 2002). Soybean plants were watered with sterilized distilled water and once in every two days supplemented with 15 ml of modified N free Hoagland solution (Hoagland and Arnon, 1950), which includes 5 mM KH_2PO_4, 2 mM $MgSO_4$, 2.5 mM $CaSO_4$ and micronutrients 46 µM H_3BO_3, 0.3 µM $CuSO_4$, 0.8 µM $ZnSO_4$, 9 µM $MnCl_2.4H_2O$, 0.1 µM $H_2MoO_4.H_2O$, and 89 µM Fe-EDTA (pH 5.5-5.8).

The plant height (20 and 55 DAP (days after planting)), nodule number (NN), pod number (PN) and the plant fresh weight (PFW) at 55 DAP were measured in each soil suspension treatment to identify the most effective soil suspension level for *Rhizobium* isolation. The leaf chlorophyll content was measured in the youngest mature leaf (55 DAP) of each soybean plant by using a hand held chlorophyll content meter 200 *(CCM200, Opti-sciences, INC, NH, USA)*. Available N (NH_4^+ and NO_3^-) in soil and Pro-mix was measured by using Kjeldahl distillation method (Bremner, 1965; Li et al., 2003).

2.3.2 *Rhizobium* Isolation

Thirty-five days after inoculation, three plants were uprooted without damaging the root system. For the isolation stage, fresh roots were collected and washed with sterilized distilled water to remove the Pro-mix and the soil particles. The nodules were detached from the roots by using a sterilized scalpel blade and then nodules were surface sterilized by immersing them in 95% C_2H_5OH for 10 seconds. Further, sterilization was carried out by soaking those nodules in a 3% H_2O_2 solution for 4-5 minutes. Finally, the nodules were five times washed with sterilized distilled water and kept in sterilized Petri plates. In order to identify the best *Rhizobial* isolation method, several techniques, as described by the Somasegaran and Hoben (1985), were practiced. They were:

1) One millilitre of sterilized distilled water was transferred into the Petri plate with nodules. The nodules were crushed using a sterilized toothpick and properly mixed. From the slurry, 0.5 ml was transferred into a Petri plate and then 15-20 ml of yeast manitol agar (YMA) with Congo red was poured into the each dish (pour plate technique). YMA consists of 0.5 g of KH_2PO_4, 0.2 g of $MgSO_4$. 7

H_2O, 0.1 g of NaCl, 0.5 g of yeast extract, 10 g of mannitol, 0.5% of Congo red solution and 15 g of agar per litre. After that, the content was mixed properly by gently moving the covered dish clockwise and counter-clockwise. Then the plates were kept in a laminar flow hood to solidify the agar. The inoculated Petri plates were incubated in an inverted position in an incubator *(307C, Cole-Parmer, IL, USA)* at 28 °C for 3-4 days until colonies appeared (Figure 2.1).

2) One millilitre of sterilized distilled water was transferred into the Petri plate containing nodules. The nodules were crushed by using a toothpick and mixed properly. Then the slurry was diluted. For this, 0.5 ml of slurry was transferred into a conical flask containing 5 ml of sterilized distilled water. From the diluent, 0.5 ml was transferred into the sterilized Petri plate and then 15- 20 ml of YMA with Congo red was poured into the each dish (pour plate technique). The plates were kept under the laminar flow hood until agar got solidified and an incubated in inverted position in the incubator *(307C, Cole-Parmer, IL, USA)* at 28 °C for 3-4 days until colonies appeared (Figure 2.1).

3) One millilitre of sterilized distilled water was transferred into the Petri plate containing nodules. The nodules were crushed by using a sterilized toothpick and properly mixed. A sterilized loop was dipped in the slurry and then streaked on the surface of YMA plates with Congo red. The plates were kept under the laminar flow hood until agar solidified and incubated in an inverted position in the incubator *(307C, Cole-Parmer, IL, USA)* at 28 °C for 3-4 days until colonies appeared (Figure 2.1).

| Technique 1 pour plate | Technique 2 pour plate | Technique 3 spread plate |
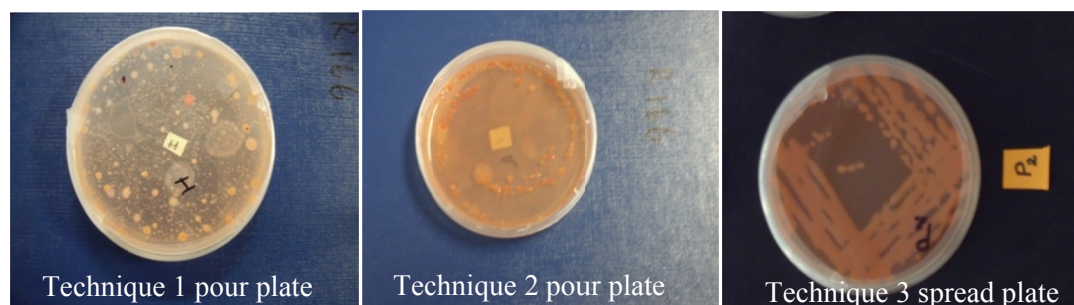
Figure 2.1 Examples of plates obtained for different *Rhizobial* isolation methods.

The colonies with no or little absorption of Congo red were selected and streaked on YMA medium for screening (Somasegaran and Hoben, 1985). The isolates were checked for typical *Rhizobial* colony morphology. The following typical *Rhizobial* colony characters were considered: round shape colonies varying from flat, domed or conical shape on agar with smooth margins, white to opaque, often gummy and soft in appearance. The potassium hydroxide (KOH) test was used to separate gram negative and gram positive bacteria. For the test, 1-2 drops of 3% KOH were placed on a clean slide and a few colonies were transferred onto the slide using a sterilized loop. The slide was kept on a dark coloured bench top and the materials on the slide were stirred for 5-10 times. The slides with viscous (liquid follows the loop) solution were considered as positive reactions which identified the gram negative bacteria (Gregersen, 1978). The positive isolates were streaked on peptone glucose agar (PGA) and YMA with Congo red or bromothymol blue (BTB). The colony morphology and the medium colours were observed over time. Generally, fast growers took 3-5 days while 5-7 days were required for slow growers (Somasegaran and Hoben, 1985). Based on the above phenomenon, the slow and fast growers were categorized.

An authentication test (Somasegaran and Hoben, 1985) was performed in the growth chamber under controlled environment to identify the ability of isolates to have soybean

nodulation. Surface sterilized pre-germinated (10 seeds) soybean seeds were sown in sterilized Pro-mix medium. After germination, the seedlings were thinned out by keeping three healthy seedlings. YMA broth cultures were prepared and morphologically identified isolates were cultured (incubated at 28 ^{o}C) until they grew. One millilitre from the each broth culture was introduced to the pre-germinated seedlings and kept in a growth chamber. The same routine practice described for the capture experiment above was followed. Nodule formation was observed at 30 DAP.

2.3.3 Statistical Analysis

The experiment was a single factor factorial, arranged in completely randomized design. Before running the ANOVA, the normality and constant variance were checked by using Minitab 15 statistical software. The independence was assumed through randomization. The PROC MIXED procedure was used in SAS 9.2 statistical software for analysis. The statistical significance criteria was a Type III error rate of $P = 0.05$ with 95% confident interval. LS means (Least Square Means) were used as the multiple mean comparison method when the effects were significant.

2.4 RESULTS

2.4.1 Effect of Soil Inoculation on Soybean Plant Growth (Capture Experiment)

Five days after sowing, the soybean seeds started to germinate. There was no difference $(P < 0.5893)$ in the plant height (Figure 2.2) at 20 DAP. However, at 55 DAP, plant height increased significantly $(P < 0.0011)$ in Pro-mix medium, compared to the soil. There were 58% and 21% increases in plant height in Pro-mix medium compared to the Wellington and the Habitant soil. The plant height of soybean grown in the Wellington soil was significantly retarded, resulting in the lowest heights among the treatments. Soil suspension did not increase the plant height compared to the water control.
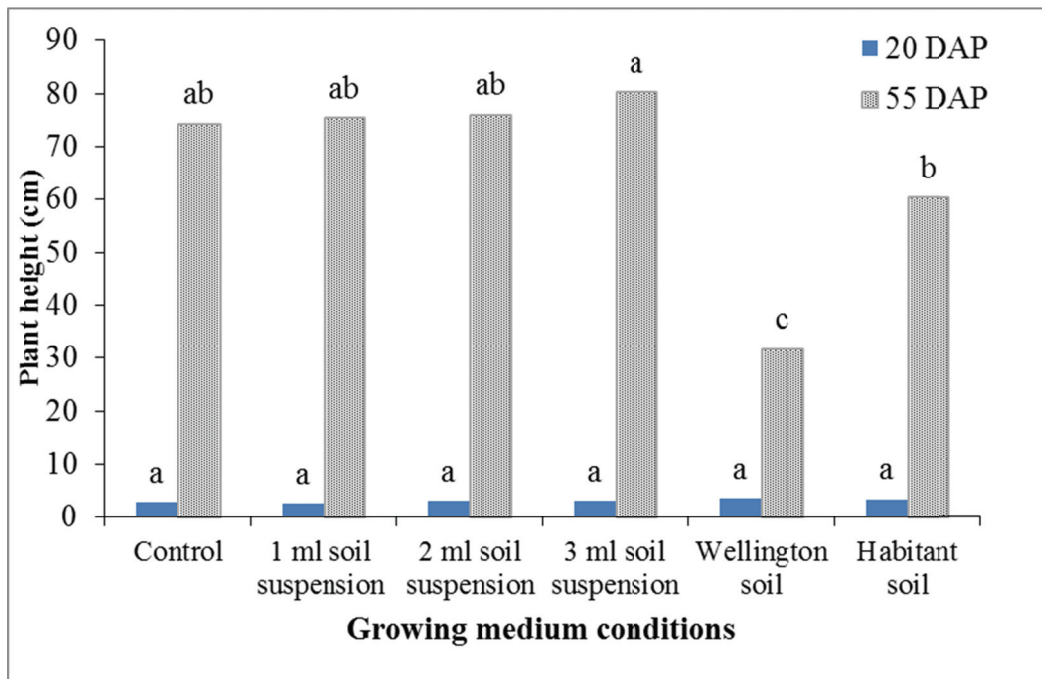
Figure 2.2 Plant heights versus growing medium conditions at 20 and 55 DAP.
Along a line, means with same letter are not significantly different $(P < 0.05)$.

Application of soil suspension from dykeland soil significantly increased $(P < 0.0244)$ the NN (Figure 2.3) compared to the control and plants grown in soil medium.

The highest number of nodules were observed in plants treated with 2 and 3 ml of soil suspension, while the plants grown on dykeland soils had the lowest number (Wellington = 5, Habitant = 4). The control plants did not yield any nodules and confirmed the non-contamination of the system.
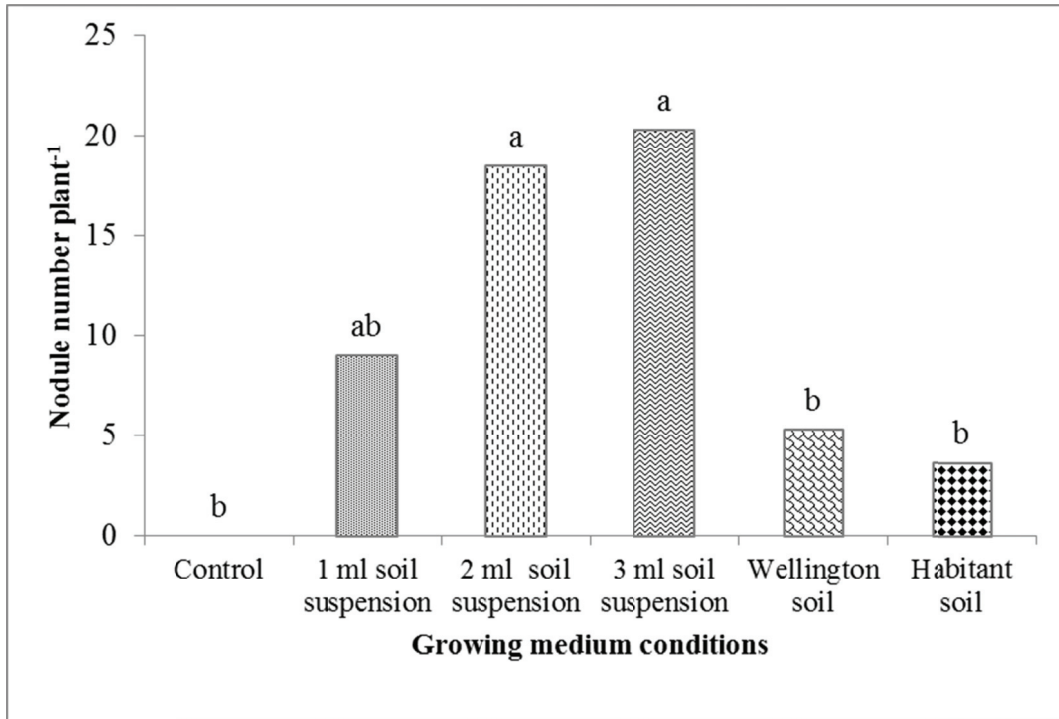
Figure 2.3 Nodule number versus growing medium conditions at 55 DAP. Means with same letter are not significantly different ($P < 0.05$).

The soil suspension rate significantly *($P < 0.0458$)* affected the pod number compared to the control plants, resulting in 33% and 16% gains in S2 and S3 plants, respectively. Low pod numbers were observed in the treatments derived from the dykeland soils above. On average, the plants treated with soil suspension rate S2 produced the highest number of pods (Figure 2.4). The rate of soil suspension did not affect PFW (Figure 2.5). The plant growth was significantly retarded in dykeland soils.

The leaf chlorophyll content was measured 55 DAP, which was presumably the peak period of soybean symbiotic N_2 fixation. The chlorophyll content was significantly greater *(P < 0.0440)* (Figure 2.6) in all treatments compared to control plants. However, there was no significant difference in chlorophyll content between soil suspension rates.
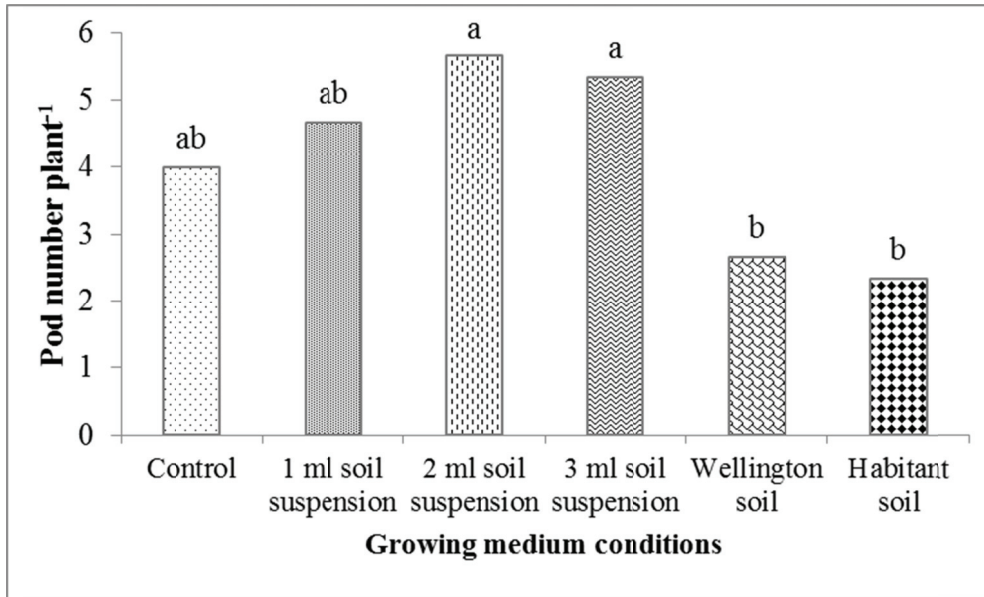
Figure 2.4 Pod number versus growing medium conditions at 55 DAP. Means with same letter are not significantly different ($P < 0.05$).
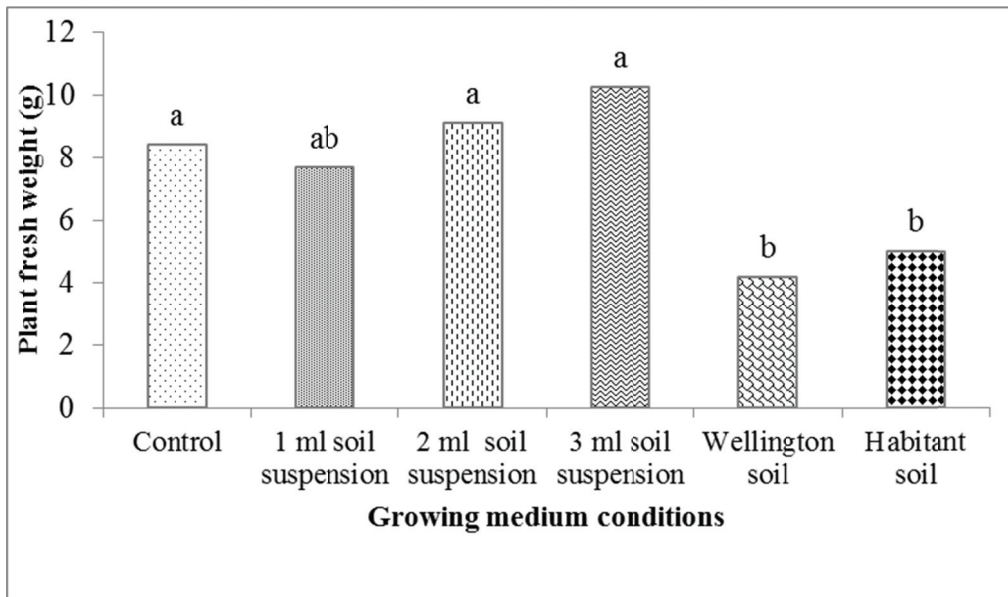
Figure 2.5 Plant fresh weights versus growing medium conditions at 55 DAP. Means with same letter are not significantly different ($P < 0.05$).
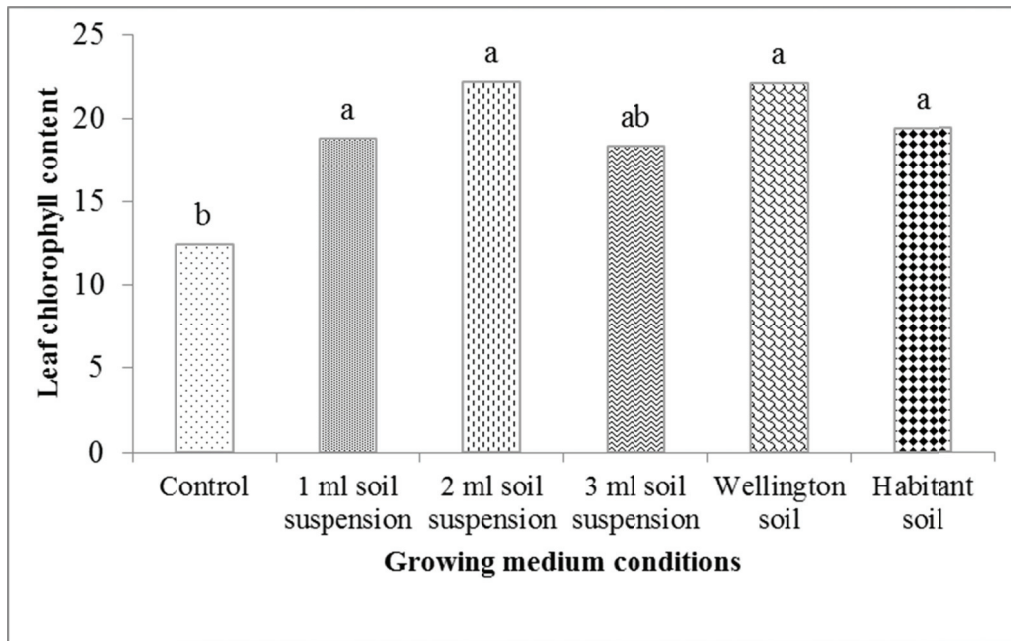
Figure 2.6 Leaf chlorophyll content versus growing medium conditions at 55 DAP.
Means with same letter are not significantly different ($P < 0.05$).

2.4.2 N Content of the Growing Medium at 55 DAP.

At the time of planting, there was a higher amount of mineral N in Pro-mix medium compared to the dykeland soil (Table 2.1). The NO_3^- level of the Pro-mix was 16% greater than in the dykeland soils. At 55 DAP, there was no significant difference in NO_3^- *(P < 0.0958)* and NH_4^+ *(P < 0.1107)* in growing media. The NO_3^- level of the Pro-mix media was remarkably lower in S0 and S1 treatments (Table 2.2). Considering plants grown in the Pro-mix medium, the control plants had taken up 45% NH_4^+ and 78% NO_3^- from the initial levels. The plants assimilated large amounts of NO_3^- from the Pro-mix medium as it was the common source of N.

Table 2.1 The available N content of growing media at seed sowing

Media	Soil NH_4^+ (mg kg^{-1})	Soil NO_3^- (mg kg^{-1})
Autoclaved pro-mix	11.2	53.2
Wellington soil	8.4	21.0
Habitant soil	8.4	19.6

Table 2.2 The available N content of growing media at 55 DAP

Treatments	Soil NH_4^+ (mg kg^{-1})	Soil NO_3^- (mg kg^{-1})
Control	6.06	14.93
1 ml of suspension	7.93	11.66
2 ml of suspension	8.40	20.06
3 ml of suspension	7.46	21.46
Wellington	4.43	12.36
Habitant	3.96	7.93

2.4.3 Identification and Characterization of Isolated *Rhizobium* Strains (Capture Experiment)

Compared to the Wellington soil, nodulation in the Habitant soil was poor. The nodules produced by the Habitant soil were smaller in diameter. At the beginning of the isolation, numerous types of colonies appeared on the primary isolate plates prepared with YMA medium. Even the plates streaked with single nodules produced more than one type of *Rhizobial* colony. The pour plate technique produced a variety of colonies while the streak plate technique produced identical colonies in the primary plates. Typical *Rhizobial* colonies were selected based on the appearance of the colonies and with little or no Congo red absorption. At the beginning, 11 types of colonies were selected from the Habitant and Wellington dykeland soils. They were denoted as W0, W1, W2, W3, W5 (from Wellington soil), H1, H2, H3 (from the Habitant soil), P1, P2 and P3 (from the Pro-mix). A greater diversity in colony appearance of isolates was observed in the Wellington compared to the Habitant soil.

In YMA medium with BTB, the blue colour is an indication of alkaline reaction, which represents the slow growing *Bradyrhizobium* species, while the yellow colour resembles the acidic reaction of fast growing *Rhizobium* species. Except for W5, the isolates changed to yellow from the colour of the BTB medium. Most of the isolated strains were fast growing *Rhizobium* species. The isolates did not grow well in the PGA medium, which is a characteristic of the *Rhizobium* species (Table 2.3).

The isolates from the Pro-mix also resembled those from the Wellington soil. Based on the KOH test W0, W1, W3, H2, H3, P1, P2, and P3 were identified as the gram negative bacteria. Colony morphological characters of W3 were similar to W2. The authentication study verified that isolates W0 had a greater ability for soybean nodule formation. The W0 was isolated from the Wellington site and formed larger active nodules in the authentication study.

Table 2.3 The characteristics of the isolated strains based on KOH test, colour change on the YMA containing BTB medium and the growth on the peptone glucose agar (PGA).

Strains	KOH test Gram status	YMA+ BTB Growth rate	PGA
W_0	G^-	yellow	NG
W_1	G^-	yellow	NG
W_2	G^-	yellow	NG
W_3	G^-	yellow	PG
W_5	G^+	Blue	NG
H_1	G^+	yellow	NG
H_2	G^-	yellow	PG
H_3	G^-	yellow	NG
P_1	G^-	yellow	NG
P_2	G^-	yellow	MG
P_3	G^-	yellow	NG

KOH test: G^+-gram positive, G^- gram negative, YMA with BTB medium: Yellow colour associate with fast growers and blue colour associate with slow growers, PGA medium: NG- no growth, PG- poor growth, MG-mild growth.

2.5 DISCUSSION

During the isolation stage, diluents were prepared with soil samples in order to reduce bacterial competition for nodulation, facilitating isolation (Yang et al., 2001). Vincent (1970) reported that for a particular legume, the number of nodule forming plants at each diluted level is correspondent to the number of bacterial cells that nodulate. In the capture experiment, the plants supplemented with different levels of diluted soil (10^{-1}) suspension produced greater number of nodules than the plants grown in the soil medium. The diluted soil suspensions (1, 2, and 3 ml) contained lesser numbers of *Rhizobia* than was found in the soil, which may reduce bacterial competition for nodulation.

In the current study, plant height, plant fresh weight, and pod number were significantly higher in Pro-mix medium than in soil medium. The major difference may be due to the amount of available N growing in the media. Greater amounts of available N in Pro-mix media facilitated plant growth and pod formation. As a result, there was a significant difference between the Pro-mix and soil media in terms of plant fresh weight, height, and pod number. Previous studies have reported that reduced symbiotic N fixation is due to high mineral N levels in the growing media (Bergersen et al., 1989; Albareda et al., 2009). Furthermore, the greater amount of available N in Pro-mix media can suppress root infection and nodule formation since the plants get sufficient amounts of N from the growing medium. In agreement with Brockwell et al (1989), the S3 and S2 soil suspension levels produced the highest number of nodules. Therefore, use of 2 and 3 ml (10^{-1}) soil suspension in the capture experiment was effective in *Rhizobial* isolation.

According to Waterer and Vessey (1993), N concentration and water holding capacity of growing media can alter the nodule number and N fixation. The dykeland soils are sandy clay loam in texture and frequently compacted with reduced water percolation. In our study, we observed lower numbers of nodules in compacted soil medium compared to the Pro-mix media. This is consistent with the results of Buttery et al (1998), who showed a reduction of the nodule number and the nodule size in sandy loam soil with high bulk density. In addition, the poor root growth associated with the soil conditions could be the other reason for the reduced nodulation and plant growth. The root volume reduction is associated with the nutrient uptake by the plant, which retarded plant growth. However, leaf chlorophyll content of plants grown in the dykeland soil was similar to the inoculated plants in the Pro-mix medium. Reeves et al (1993) reported that there was a good correlation between chlorophyll content and leaf nitrogen content. Therefore, it can be concluded that the nodules formed in the plants grown in the soil medium fixed N effectively. The leaf chlorophyll content of the control plants in Pro-mix medium was significantly low. This indicates that they have not obtained the advantage of the higher NO_3^- content in the Pro-mix medium.

Soil conditions and the number of nodulating *Rhizobia* in the soil were identified as the main factors for multiple nodule occupancy (May and Bohlool, 1983). In addition, are considerable amount of dual nodule occupancy was reported under in vitro conditions than in the field (Pinochet et al., 1993: Palaniappan et al., 1997). More than one *Rhizobium* species was isolated from a nodule during the capture experiment in this study.

The majority of isolates in this study were fast growers. The soil samples were obtained from non-tilled soils during the spring. Coutinho et al (1999) reported that the majority of isolates from non tilled soils are fast growers. However, in the Habitant site, the soil was poorly enriched with *Rhizobia* compared to the Wellington soil. This may be due the variability in soil conditions and soil management practices, which can greatly influence the soil *Rhizobial* profile. Solid dairy manure was added to the Wellington field and therefore, the soil biological properties were likely enhanced. Application of dairy manure increased the soil organic carbon content, resulting in an increase in the microbial biomass. Further, Zengeni and Mpepereki (2003) reported that manure application augmented the *Rhizobial* population, acting as an energy source. The Habitant soil has inherent soil salinity (see chapter V), which reduced the survival of previously introduced *Rhizobia* and limited the *Rhizobial* population to saline tolerant strains.

2.6 CONCLUSION

The isolation and identification of existing soil *Rhizobia* from dykeland soils can be beneficial in terms of promoting and improving soybean symbiotic N fixation. Depending on the dykeland soil conditions, there was a great variability in isolates. The majority of the isolates were fast growers. The isolate W0 had the greatest ability for soybean nodulation in the authentication trials. Use of a growing medium with greater available N levels for *Rhizobium* isolation can alter the observations and results of isolation studies. However, further investigations are necessary to confirm the effective N fixation ability of these isolates in the growth chamber, as well as in the dykeland fields.

CHAPTER 3   SYMBIOTIC NITROGEN FIXATION AND GRAIN YIELD OF SOYBEAN IN RELATION TO *BRADYRHIZOBIUM* INOCULATION AND NITROGEN USE IN ACIDIC DYKELAND SOIL

3.1. INTRODUCTION

Soybean plants utilize both N fixed in the root nodule and N absorbed from the soil and fertilizer (Wery et al., 1986). It was reported that in an agricultural system, the maximum soybean yield can be obtained by optimum use of symbiotic N fixation and the mineral N uptake. At the early growth stages, the soybean plant mainly depends on the mineral N assimilation and in later stages, on symbiotic N fixation (Harper, 1974).

There are several studies focused on soybean inoculation and inorganic fertilizer N responses at different growth stages and climatic conditions. It was reported that *Bradyrhizobium* inoculant promoted soybean grain yield (Albareda et al., 2009). Furthermore, there should be 10^5-10^6 *Rhizobial* cells per seed to maximize the grain yield (Catroux et al., 2001). It was found that under favourable soil conditions, N fertilization is not necessary for inoculated soybean (Welch et al., 1973; Duong et al., 1984; Schmitt et al., 2001; Barker and Sawyer, 2005; Diaz et al., 2009). However, other studies showed that the symbiotic N fixation is not adequate to fulfill the soybean N demand and fertilizer application is necessary (Gan et al., 2003; Osborne and Ridell, 2006; Ray et al., 2006; Caliskan et al., 2008).

In Nova Scotia, there are about 18,000 ha of dykelands and soybean is one of the field crops grown on these lands. However, there is a lack of information about soybean symbiotic N fixation performance under dykeland conditions with respect to *Bradyrhizobium* inoculant and starter N fertilizer on an acidic dykeland site.

3.2 OBJECTIVES

The aims of this study were to investigate the effect of inoculant and starter N fertilizer on soybean symbiotic N fixation as well as grain yield under dykeland conditions.

3.3 MATERIALS AND METHODS

3.3.1 Site Description

The experiment was conducted during the summer of 2010 in the Wellington dykeland, located in the Annapolis valley in Nova Scotia. The soil type is an Acadia marine loam (silty clay loam in texture) whose soil classification is referred to in Appendix A. According to the previous year (fall 2009) soil analysis, the chemical properties of the soil (depth of 0-15 cm) were as follows: 8.4 mg NH_4^+, 21 mg NO_3^-, 100 mg of phosphorous, 200 mg of potassium per kilogram of soil and pH of 4.8. Nova Scotia has a modified continental climate with a mean annual rainfall of 1250 mm. The land was subjected to crop rotation, which in the first year was soybean followed by corn in the second year, followed by grass in the third year (corn -grass-soybean). The field was cultivated with corn the previous year.

3.3.2 Field Experimental Setup

In the field, the treatments were assigned in a split plot design with three replications where the main plot treatments were arranged in an RCBD. The treatments consisted of four rates of *Bradyrhizobium japonicum* inoculant and four rates of starter N fertilizer. The levels of inoculant used were: 0 (IR0), 1.5 (IR1.5), 3 (IR3) and 4.5 (IR4.5) g kg^{-1} seed. The standard rate of commercial inoculant 'Nitragin' for soybean is 3 g kg^{-1}

of seed. The levels of N applied were; 0 (N0), 10 (N10), 20 (N20) and 30 (N30) kg N ha^{-1}. The N0 is the recommended N fertilizer level used in NS. To minimize cross contamination, the different rates of inoculant were used as main plots, while N fertilizer treatments were sub-plots. Different inoculant levels were randomly assigned to the four main plots in each block and the N fertilizer treatments were randomly assigned to the subplots within each main plot (Figure 3.1).

The size of a sub plot was 2 m × 4 m (area of 8 m^2). Inter and intra row spacing was 17.5 cm and 9.5 cm, respectively. There was a 0.5 m of buffer zone in between two sub-plots. There were total of 12 (3× 4) main plots and 48 (4× 4× 3) sub-plots in this study.

Block/Rep 1

IR_0					$IR_{4.5}$					IR_3					$IR_{1.5}$			
N_{10}	N_0	N_{20}	N_{30}		N_{30}	N_{10}	N_{20}	N_0		N_0	N_{10}	N_{30}	N_{20}		N_{20}	N_{30}	N_{10}	N_0

Block/Rep 2

$IR_{1.5}$					IR_3					$IR_{4.5}$					IR_0			
N_{10}	N_{30}	N_0	N_{20}		N_{30}	N_{20}	N_0	N_{10}		N_{10}	N_0	N_{20}	N_{30}		N_{20}	N_{10}	N_0	N_{30}

Block/Rep 3

IR_0					IR_3					$IR_{1.5}$					$IR_{4.5}$			
N_{20}	N_0	N_{10}	N_{30}		N_0	N_{10}	N_{20}	N_{30}		N_{30}	N_{20}	N_{10}	N_0		N_{10}	N_0	N_{20}	N_{30}
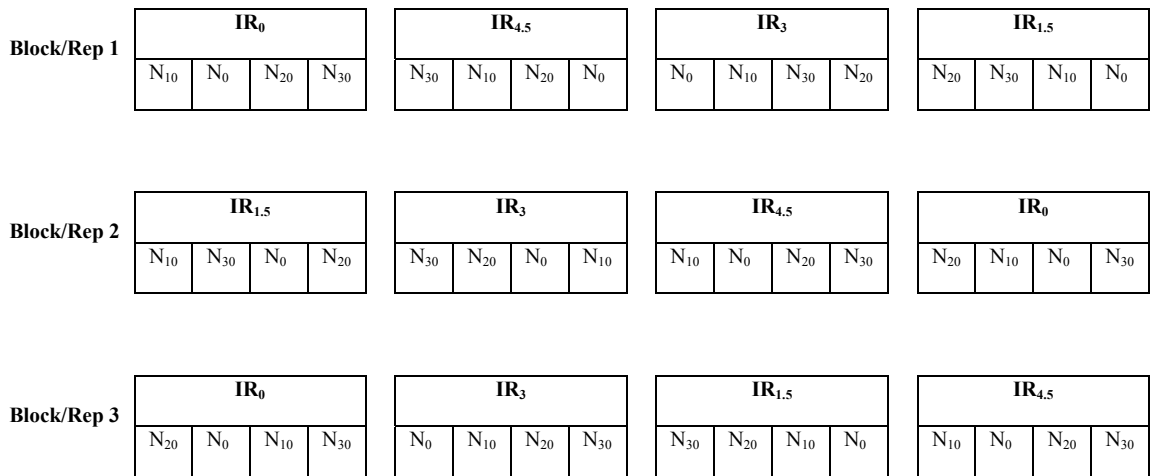
Figure 3.1 Field experiment in the Wellington dykeland

Seeds of genetically modified Soybean cultivar "LynX" were used. This cultivar was selected specifically due to superior performance in the Maritime region. Because of its high yield, "Lynx" has been ranked number one in the Maritime trials. Commercial peat based inoculant "Nitragin" was used as the *Bradyrhizobium japonicum* inoculant source. To ensure the adhering of bacteria to the seed surface, seeds were mixed with the

inoculant prior to sowing. The slurry of inoculant was prepared by using 2 ml of distilled water (H_2O) and uniformly mixed with the seeds. The field planting was carried out by hand on May 31, 2010 at a rate of 20, 0000 seeds ha^{-1}. Seed sowing was performed prior to a rainy period, according to the weather report of Environment Canada (www.weatheroffice.gc.ca). First, the seeding was carried out in un-inoculated (IR0) plots to prevent contamination with inoculated seeds. Since the selected starter N levels were low in quantity, they could be easily lost from the soil with early application. Therefore, ten DAP, N rates (0, 10, 20, and 30 kg ha^{-1}) were broadcast as ammonium nitrate (NH_4NO_3). As the potassium (K) source, murate of potash (KCl) was applied (K=22 kg ha^{-1}, based on the previous year testing) to each sub-plot. At the beginning, the weeding was manual; Round up (Glyphosate) was applied later in the early pod filling stage due to the severe weed infestation. At the vegetative stage, the herbicides were not applied as there was no severe weed problem.

3.3.3 Soybean Field Plant Sampling

Seedling emergence occurred ten days after the sowing. Soybean plant samples were obtained at three different growth stages. The plant sampling stages were R2, R5 and R7 (Table 3.1). At R2 and R5 stages, ten plants were randomly uprooted from each plot, while 20 plants were uprooted at R7. The plants and the rhizosphere soils were gently uprooted with the help of a spade and the soil was removed carefully.

Table 3.1 Soybean plant-sampling stages

Days after sowing	Growth stage
60	Full flower (R2)
105	Begin seed (R5)
132	Harvest maturity (R7)

Since the extraction of root bleeding sap was difficult to perform at the field level, the soybean stem segments were used to quantify the N fixation. The ureide-N in stems is insensitive to diurnal fluctuations and unchanged by temperatures of 20-30 ^{o}C (Herridge, 1982). Therefore, the temperature of the uprooted plant materials was maintained at 20-30 ^{o}C (ambient temperature) until they were processed. Three plants from the uprooted sample were partitioned into leaves, petioles, stem, pods, and nodulated roots for the analysis of ureide-N and NO_3-N at R2 and R5 stages. The plant fresh weight (PFW), plant height, root length (the length from shoot base to main root tip), nodule number, nodule weight, pod number and fresh pod weight were obtained from the three randomly selected plants. The plant samples were dried for 48 hours in a conventional oven at 60 ^{o}C. The dried stem parts were ground using a Wiley mill and passed through the 60 mesh size (1 mm) screen and stored until analysis.

The sap nitrate (NO_3-N) and K concentrations in soybean leaves, petioles, stem were measured at both R2 and R5 stages, and pod sap was measured at R5 stage. The sap was collected using a handheld plant sap presser *(Spectrum Technologies, IL, USA)*. The sap NO_3-N and K were measured with a cardy nitrate meter *(Spectrum Technologies, IL, USA)* and a cardy potassium meter *(Spectrum Technologies, IL, USA)*.

At the R7 stage, the harvested plants were divided into seeds, pod walls and stems and the dry weight of each component was determined by oven drying at 60 ^{o}C until a

constant weight was obtained. Harvest index (HI) was calculated using the fraction of the seed, which contributed to the total biomass.

3.3.4 Soil Sampling

Soil samples were obtained at the R2 stage to determine the effect of applied N fertilizer. Soils samples were collected randomly (mostly from the front, middle and rear sections of the plot) at a depth of 0-15 cm, using an Edmonton auger. The representative samples for the chemical analysis were obtained from composite soil samples. Soil moisture was determined by oven drying 10 g of fresh soil at 105 ^{o}C for 48 hours. The rest of the soil samples were air dried and debris was removed, sieved through a 2 mm mesh size and stored for later chemical analysis.

3.3.5 Plant Tissue Chemical Analysis

To determine the plant total N concentration, the plant samples were acid digested and distilled by using the Kjeldahl distillation method (Bremner and Mulvaney, 1982). It was then titrated with 0.01 N hydrochloric acid (HCl).

The N fixation was quantified by the method suggested by Herridge (1982). Distilled water was used to extract the ureide-N and NO_3-N compounds from the ground stems. The ureide-N and NO_3-N were then colorimetrically analyzed.

3.3.5.1 Determination of Plant Total N

The Labconco digestion unit *(Rapid digestor, Labconco cooperation, Kansas City, Missouri)* was employed to digest the soybean plant samples. Ground plant tissue samples of 0.5 g were weighed and 10 ml of concentrated sulphuric (H_2SO_4) acid was added to a digestion tube, followed by placing on a preheated (300 ^{o}C) digestion block for 40 minutes in the fume hood. Digestion tubes were removed from the digestion block and cooled for 5 minutes. Then, 2 ml of 30% hydrogen peroxide (H_2O_2) was mixed with the partially digested tissues sample and kept on the digestion block for another 15 minutes. Again, the sample was removed from the digestion block and cooled for 5 minutes, after which 2 ml of H_2O_2 was added. This step was repeated until the digestion solution was colourless. Once the process was completed, the digested plant tissues were kept under the fume hood for 30 minutes. Then the solution was transferred into a 50 ml volumetric flask and volumerized by using distilled water. The diluted digestion solution was employed for plant total N analysis, as described below.

Total N content was determined by the Kjeldahl distillation method in the Labconco distillation unit *(Model 64132, Labconco cooperation, Kansas City, Missouri)*. Ten millilitres of the digested solution were transferred into a distillation tube and mixed with 10 ml of 40% (10 N) sodium hydroxide (NaOH) solution. It was then distilled until the volume of the receiving flask, which contained 5 ml of boric acid (H_3BO_3) and 5 drops of mixed indicator (methyl red and green bromocresol), doubled (approximately 7 minutes). Finally, the distillate was titrated against 0.01 N HCl until the colour became pinkish grey. The total plant N was calculated as N mg plant^{-1}.

3.3.5.2 Determination of Stem Ureide Compounds

a) Extraction of ureide compounds

The ureide compounds were extracted from the dried stem tissues (Herridge, 1982). A ground stem tissue sample of 0.5 g was mixed with 25 ml of distilled water in a boiling tube and heated for 1-2 minutes in a boiling water bath. The suspension was filtered into a 50 ml of volumetric flask by passing it through Whatman No: 40 filter paper in a funnel. The residues were washed onto the filter and rinsed with the distilled water. When the content cooled, the filtrate was volumerized into 50 ml by using the distilled water. The concentration of ureide-N was measured as per the Young and Conway (1942) method and NO_3-N by the Cataldo method (Cataldo et al., 1974).

b) Ureide assay

From the stem extract, 0.5 ml was taken and transferred into a test tube and mixed with 2 ml of distilled water (1:5 dilutions). The diluted solution was mixed with 0.5 ml of 0.5 N NaOH and kept in a boiling water bath for 10 minutes. One millilitre of ice-cooled 0.65 N HCl /phenylhydrazinium solution was added into the reaction mixture and kept in a boiling water bath for another 2 minutes. Then, the test tubes were removed from the boiling water bath and immediately placed in an ice bath for 15 minutes. Finally, 2.5 ml of ice-cooled potassium ferricyanide (HCl/K_3FeCN_6) were added. The solution was allowed to rest for 10 minutes for optimum colour development. The absorbance was measured at 525 nm by the spectrophotometer *(Ultrospec 2100 pro UV/Visible Spectrophotometer, Biochrom Ltd, CB, UK)*. Standard solutions of ureide (0, 0.01, 0.02,

0.04, and 0.1 mM) were prepared by using 1 mM of allantoin stock solution. Based on the standard curve, the stem ureide concentrations were determined.

c) Nitrate assay

From the stem extract, 0.05 ml was transferred to the test tube and mixed with 0.20 ml of 5% salicylic/sulphuric acid solution and left on the bench for 20 minutes. Then, 4.75 ml of 2 N NaOH was added and kept for 10 minutes for optimum colour development. The absorbance at 410 nm was measured by the spectrophotometer *(Ultrospec 2100 pro UV/Visible Spectrophotometer, Biochrom Ltd, CB, UK)*. Standard solutions of NO_3^- (0, 1.25, 2.50, 5.00, 10.00, and 15.00 mM) were prepared by using 25 mM standard potassium nitrate (KNO_3) stock solution. The NO_3^- concentrations in the stems were calculated from a standard curve.

d) Calculation of daily N fixing activity and daily N absorption rate

The relative abundance of ureide was determined by using the following equation described by Herridge (1982).

$$\% \text{ of N derived from atmospheric } N_2 \text{ (RU\%)} = \frac{4 \times \text{Ureide-N}}{[4 \times \text{Ureide-N} + \text{Nitrate-N}]} \times 100$$

The average of daily N fixation rate and N absorption rate over the growth stages were estimated according to the method described by Tewari et al (2004). To calculate the daily N gain, the total N determined by the Kjeldahl digestion method was used. The total N for each sampling date was assigned as N_1 (R2) and N_2 (R5) and corresponding

sampling dates as D_1 (R2=65 DAP) and D_2 (R5=105 DAP). D_0 was designated as the date of sowing. The average daily N gain (ΔN) was estimated as follows:

$$\Delta N_{2\text{-}1} = N_2 - N_1 / D_2 - D_1$$

The total amount of N assimilated (uptake + N fixation) by the plant was assumed to be the same as the RU%. The average RU% was calculated as,

$$RU\%_{2\text{-}1} = (RU\%_1 + RU\%_2)/2.$$

From sowing to the first date of sampling (D_1), the average RU% $_{0\text{-}1}$ was assumed to be RU%$_1$. The daily N fixing rates and daily N absorption rates were calculated as below:

Daily N fixation rate = $(\Delta N_{2\text{-}1} \times RU\%_{2\text{-}1} / 100) / D_2 - D_1$

Daily N uptake rate = $[\Delta N_{2\text{-}1} \times (100 - RU\%_{2\text{-}1}) / 100)] / D_2 - D_1$

3.3.6 Soil Chemical Analysis

Soil available N (NH_4^+ and NO_3^-) was measured with the Kjeldahl distillation method (Bremner, 1965; Li et al., 2003). Soil pH (1: 2.5, water) and electrical conductivity (EC) (1: 1) were measured by using a combined pH and conductivity meter *(Model 3540, Jenway, Bibby scientific Ltd., ST, UK)*.

To extract the soil available N, 10 g of soil were mixed with 25 ml of 2 N KCl. The solution was placed on a rotating shaker *(Model KS, 130 CS 1, IKA, KS, USA)* and agitated at 180 rpm for 30 minutes. The extract was filtered through a Whatman No: 40 filter paper. To determine the soil NH_4^+-N, 10 ml from the aliquot were transferred to a distillation flask and 0.2 g of magnesium oxide (MgO) and calcium chloride ($CaCl_2$) were added. The distillation flask was connected to the Labconco distillation apparatus *(Model*

64132, Labconco cooperation, Kansas City, Missouri). The released NH_4^+ was collected to a receiving flask which contained 2% boric acid (H_3BO_3) and 5 drops of mixed indicator (methyl red and bromocresol green). The distillation was carried out for 6 minutes. Once the distillation was completed, the receiving flask was removed. In order to determine the soil available NO_3^-N, 0.2 g of Devarda alloy was subsequently added to the distillation flask and distilled for 6 minutes. The ammonium borate trapped in the receiving flask was titrated by using 0.01 N HCl until the colour changed from greenish blue into pinkish gray. The soil available N was calculated using the HCl acid volume.

3.3.7 Statistical Analysis

Before running the ANOVA, the normality and constant variance were checked with the Minitab 15 statistical software. The independence was assumed to be through randomization. The PROC MIXED procedure in SAS 9.2 statistical software was used for the data analysis of each variable obtained in the experiment. The block and the interaction between the block and inoculant were considered as random effects. The rate of inoculant and the rate of starter N were considered to be fixed effects. The statistically significant criterion was a Type III error rate of $P = 0.05$, with 95% confidence interval. When the interaction effects were significant, the multiple mean comparison method of LS means (Least Square Means) was used. If the main treatment effects (rate of inoculant and rate of starter N) were significant, LSD (Least Square Difference) analysis was computed. To evaluate the differences in treatment means, orthogonal contrasts were constructed for the inoculant and the N fertilizer comparison. For all the measured variables, the contrasts were determined by comparing the different levels of inoculant and starter N rates.

3.4 RESULTS

3.4.1 Effects of *Bradyrhizobium* Inoculant and Starter N on Soybean Plant at the Vegetative Stage

There was 85% seed emergence in the Wellington field. Flowering and pod elongation was observed at 45 and 60 DAP, respectively. Early flower initiation was observed in N fertilized plots. The field was subjected to rapid moisture fluctuations throughout the growing season (dried and water logged conditions depending on precipitation).

At 60 DAP, the plants were at early pod filling stage (R2). There was no significant interaction effect between the rates of inoculant and the rates of fertilizer N on PFW and plant dry weight (PDW) at this stage (Table 3.2). The effect of starter N fertilizer was not significant on PFW, PDW, plant height, and nodule weight at 60 DAP. The height of the inoculated plants was significantly greater than un-inoculated plants.

There was a significant increase in the root length of inoculated plants, while un-inoculated plants had the lowest root length. The effect of starter N on soybean root length was not significant (Table 3.2).

Table 3.2 Soybean plant fresh weight (PFW), dry weight (PDW), plant height, root length, nodule number, and nodule weight of soybean as affected by inoculation and starter N rates at Wellington dykeland at 60 DAP

Means Source	PFW (g)	PDW (g)	Plant height (cm)	Root length (cm)	Nodule number	Nodule fresh weight (g)
I_0	34	9	28	13	16	0.41
$I_{1.5}$	50	16	37	15	38	1.07
I_3	52	17	39	14	32	1.08
$I_{4.5}$	43	13	37	14	31	1.02
LSD (5%)	26	7	7	2	7	0.26
N_0	40	13	36	14	30	1.01
N_{10}	47	14	35	14	31	0.94
N_{20}	46	15	34	14	29	0.83
N_{30}	47	13	36	14	27	0.81
LSD (5%)	26	7	7	2	7	0.26
Variation				*F test values*		
I	0.5308	0.2742	0.0235*	0.0407*	0.0028**	0.0085**
N	0.3867	0.6802	0.9510	0.9503	0.3305	0.3267
I× N	0.4236	0.2109	0.9322	0.6014	0.0011*	0.4744
Contrasts						
I_0 vs $I_{1.5}$,I_3& $I_{4.5}$	0.2157	0.0867	0.0297*	0.0169*	0.0006**	0.0014**
$I_{1.5}$ vs I_3& $I_{4.5}$	0.8351	0.8250	0.7325	0.1756	0.0808	0.8366
I_3 vs $I_{4.5}$	0.5132	0.4271	0.5490	0.3227	0.5782	0.6813
N_0 vs N_{10},N_{20}& N_{30}	0.0930	0.6893	0.5524	0.5855	0.4603	0.1405
N_{10} vs N_{20}&N_{30}	0.8361	0.8451	0.7157	0.8452	0.1407	0.2685
N_{20} vs N_{30}	0.8098	0.2615	0.3121	0.9451	0.4048	0.8666

*and ** significant at 5 and 1% probability level, respectively.

The nodule number and the nodule weight were significantly greater in inoculated plants than un-inoculated plants. The interaction between the inoculant and the N treatments was significant for the nodule number (Figure 3.2). The greatest nodule number was observed with IR1.5 with N20. However, higher numbers of nodules were also observed in the treatment combinations of IR1.5-N0, IR1.5-N10, IR3- N20, and IR4.5- N10.
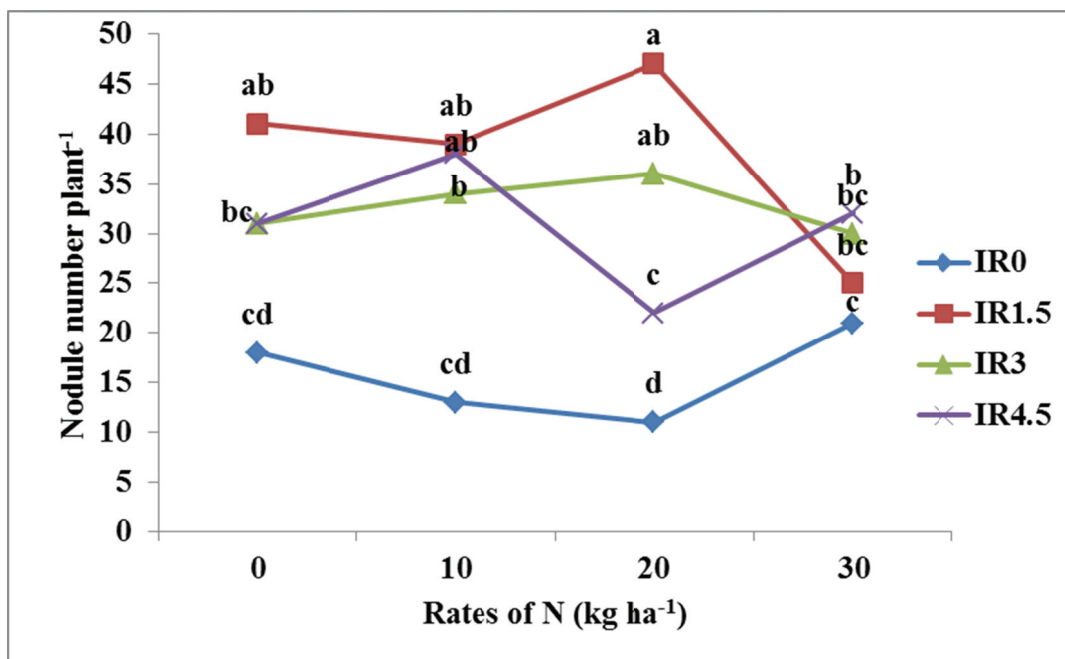
Figure 3.2 The interaction effect of rate of inoculant and rate of N on soybean plant nodule number. (Means with same letter are not significantly different at $P < 0.05$).

The nodule numbers of IR1.5 and IR3 plants were reduced with N fertilization at the N30 level. There was a substantial reduction in nodule size of IR3 and IR4.5 compared to the IR1.5 plants at N30. Noticeable amount of nodules in the control plant indicated the presence of indigenous *Rhizobia* or survivors of previously applied inoculant in the field 2 years ago. Most of the nodules formed by the control plants were located on the lateral roots; they were small in size and appeared to be ineffective. Unlike the nodule number, the nodule weights did not show a similar pattern of treatment interactions. The nodule weights of the inoculated plants were significantly higher than those of the control plants.

3.4.2 Effects of *Bradyrhizobium* Inoculant and Starter N on Soybean Plant at the Seed Filling Stage

Grain development was observed at 105 DAP. At this stage, the interaction between the inoculant and starter N rates was not significant on the PFW and the PDW. Furthermore, starter N fertilizer did not increase the PFW at 105 DAP.

Table 3.3 Soybean plant fresh weight (PFW), dry weight (PDW), pod number, pod fresh weight, and pod dry weight as affected by inoculation and starter N rates at 105 DAP

Means Source	Plant Fresh weight(g)	Plant Dry weight (g)	Pod Number	Pod Fresh weight (g)	Pod Dry weight (g)
I_0	83	28	40	50	14
$I_{1.5}$	132	42	56	77	22
I_3	145	45	57	80	24
$I_{4.5}$	128	41	54	73	22
LSD (5%)	34	12	13	20	7
N_0	117	36	50	66	19
N_{10}	114	37	51	67	20
N_{20}	129	40	51	70	21
N_{30}	130	42	55	76	22
LSD (5%)	34	12.	13	20	7
Variation			*F- test values*		
I	0.0178*	0.0322*	0.0517*	0.0380*	0.0570*
N	0.4773	0.5560	0.8254	0.6302	0.7717
I× N	0.5496	0.6104	0.4792	0.4984	0.7173
Contrasts					
I_0 vs $I_{1.5}, I_3$ & $I_{4.5}$	0.0037**	0.0062**	0.0103**	0.0076**	0.0124**
$I_{1.5}$ vs I_3 & $I_{4.5}$	0.7385	0.8571	0.8500	0.9360	0.5905
I_3 vs $I_{4.5}$	0.2487	0.4593	0.5345	0.4183	0.4788
N_0 vs N_{10}, N_{20} & N_{30}	0.4690	0.3770	0.5916	0.4664	0.4858
N_{10} vs N_{20} & N_{30}	0.1688	0.3342	0.7304	0.4209	0.5369
N_{20} vs N_{30}	0.9091	0.5617	0.4946	0.4702	0.6346

*and ** significant at 5 and 1% probability level, respectively.

The inoculated PFW and PDW were significantly high, compared to the control plants. PN and pod dry weight (PODW) were marginally significant in the inoculated

plants, compared to the control plants, while pod fresh weight (POFW) was significantly high in inoculated plants (Table 3.3). However, these effects were not significant between IR1.5, IR3 and IR4.5.

3.4.3 Effects of *Bradyrhizobium* Inoculant and Starter N on Soybean Grain Yield

The soybean harvest was taken at 132 DAP (physiological maturity stage). The interaction effect of the rates of inoculant and starter N fertilizer on soybean grain yield was not significant. However, the grain yields of inoculated plants were significantly increased (Figure 3.3) compared to the un-inoculated plants (2420 kg ha^{-1}). An increasing trend in the seed yield was observed from IR1.5 to IR4.5 inoculant rates. The seed yield was vary as IR4.5 (3666 kg ha^{-1}) and IR3 (3404 kg ha^{-1}), followed by IR1.5 (3164 kg ha^{-1}). Application of starter N fertilizer did not increase the soybean grain yield.

The seed weight (SW), residual biomass (RBM) (stems+ pod wall) and the total biomass (TBM) (seeds +stems+ pod wall) were significantly higher in the IR1.5, IR3, and IR4.5 than in un-inoculated plants (Table 3.4). Compared to the un-inoculated plants, there was an average of 42% (IR1.5), 64% (IR3), and 64% (IR4.5) increase of TBM in the inoculated plants. According to the contrast analysis, inoculant rate IR3 and IR4.5 were marginally significantly greater than IR1.5. The RBM of inoculated treatments was 76% (IR3), 70% (IR4.5), and 49% (IR1.5) higher than in the un-inoculated plants.
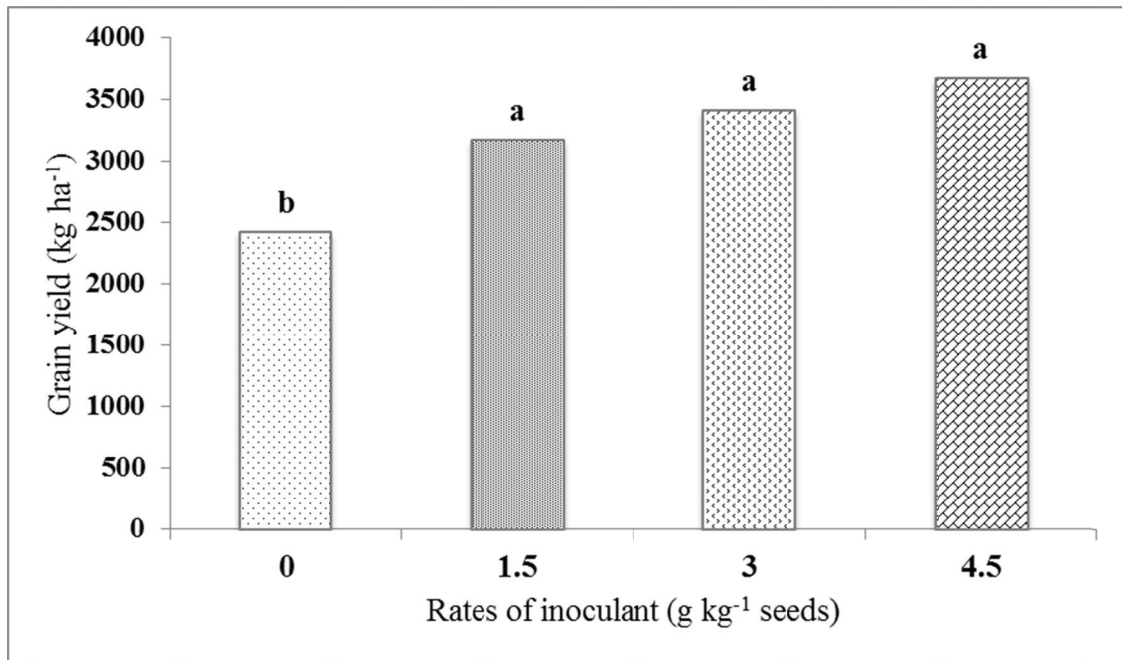
Figure 3.3 Soybean grain yield (kg ha^{-1}) at harvest as affected by rates of inoculant (LSD = 636.96; α =0.05). Means with same letter are not significantly different (*P* < 0.05).

The N0 vs. N10, N20 & N30 contrast was significant for TBM and the RBM. There were neither interaction effect, nor main effects, on the rate of inoculant and the starter N fertilizer on HI. Greater HI was observed for IR0 and N0 plants. This may be due to the reduction in plant growth at the vegetative stage.

Table 3.4 Soybean seed weight, residual biomass, total biomass, and HI as affected by inoculation and starter N rates at Wellington dykeland at harvest

Means	Seed weight ($g\ m^{-2}$)	Residual biomass ($g\ m^{-2}$)	Total biomass ($g\ m^{-2}$)	HI%
Source				
I_0	242	470	712	36
$I_{1.5}$	316	699	1016	31
I_3	340	829	1169	29
$I_{4.5}$	367	800	1167	32
LSD (5%)	64	124	154	6
N_0	296	596	891	34
N_{10}	314	713	1027	31
N_{20}	321	737	1058	32
N_{30}	335	752	1087	31
LSD (5%)	64	124	154	6
Variation		*F- test values*		
I	0.0488*	0.0039**	0.0025**	0.2209
N	0.5300	0.0668*	0.0650*	0.5991
I× N	0.8463	0.1981	0.1849	0.9452
Contrasts				
I_0 vs $I_{1.5}$,I_3& $I_{4.5}$	0.0126*	0.0009**	0.0005**	0.0639
$I_{1.5}$ vs I_3& $I_{4.5}$	0.2619	0.0721	0.0547*	0.8754
I_3 vs $I_{4.5}$	0.4769	0.6551	0.9747	0.4278
N_0 vs N_{10},N_{20}& N_{30}	0.2166	0.0103*	0.0110*	0.2077
N_{10} vs N_{20}&N_{30}	0.5477	0.5616	0.4878	0.7477
N_{20} vs N_{30}	0.5986	0.8057	0.6953	0.7261

*and ** significant at 5 and 1% probability level, respectively.

3.4.4 N Uptake Pattern of Soybean Plant at Different Growth Stages

3.4.4.1 Effects of Rate of Inoculant and Starter N on Soybean Plant Total N

At the full flower stage (60 DAP), the soybean plant total N was not affected by the rates of inoculant nor starter N (Table 3.5). All three rates of inoculant had a significantly higher plant total N content at the grain filling stage (105 DAP).

At harvest (132 DAP), the interaction effect between the rates of inoculant and the rates of fertilizer N was not significant on soybean residual biomass N (RBMN) or the

total biomass N (TBMN) or the seed N but, seed N, RBMN and TBMN were significantly high in inoculated plants (Table 3.6).

Table 3.5 Soybean total plants N as affected by inoculation and starter N rates at 60 and 105 DAP at Wellington.

Means	Plant N at 60 DAP	Plant N at 105 DAP
Source	g plant^{-1}	g plant^{-1}
I_0	0.20	0.85
$I_{1.5}$	0.29	1.30
I_3	0.34	1.33
$I_{4.5}$	0.29	1.24
LSD (5%)	0.15	0.32
N_0	0.25	1.15
N_{10}	0.28	1.14
N_{20}	0.31	1.23
N_{30}	0.28	1.21
LSD (5%)	0.15	0.32
Variation	*F- test values*	
I	0.4599	0.0515*
N	0.4738	0.9066
I× N	0.9166	0.7033
Contrasts		
I_0 vs $I_{1.5}$,I_3& $I_{4.5}$	0.1631	0.0101*
$I_{1.5}$ vs I_3& $I_{4.5}$	0.7882	0.9258
I_3 vs $I_{4.5}$	0.5737	0.5795
N_0 vs N_{10},N_{20}& N_{30}	0.1892	0.7432
N_{10} vs N_{20}&N_{30}	0.6662	0.5158
N_{20} vs N_{30}	0.4567	0.9405

*and ** significant at 5 and 1% probability level, respectively.

The TBMN was significantly higher in inoculant treatments than in the un-inoculated treatment. RBMN was significantly higher in IR4.5 (56.77 kg ha^{-1}) and IR3 (49.82 kg ha^{-1}) than in the IR1.5 (39.89 kg ha^{-1}). The starter N application did not affect the soybean seed N nor the TBMN content. However, TBMN and RBMN in fertilizer treatments were greater than IR1.5 treatment (Table 3.6). The RBMN content was significantly high in starter N rates of N10, N20 and N30 than in N0. The N Harvest Index (NHI) was significantly higher in IR3 and IR1.5 (82%) than IR4.5 and IR0, which

had 79% of NHI. The HI did not vary in N fertilized treatments, compared to the N0 treatment. Although N treatments had greater levels of TBMN, the amount of N content in the seed did not increase. As a result, there was no significant difference in NHI in any of the N fertilized treatments.

Table 3.6 Soybean seed N, residual biomass N (RBMN), total biomass N (TBMN) and N harvest index (NHI%) as affected by inoculation and starter N rates at harvest at Wellington.

Means	Seed N kg ha^{-1}	RBMN kg ha^{-1}	TBMN kg ha^{-1}	NHI%
Source				
I_0	119	30	149	79
$I_{1.5}$	167	40	207	82
I_3	182	50	232	82
$I_{4.5}$	196	57	252	79
LSD (5%)	33	9	37	2
N_0	155	36	191	80
N_{10}	165	45	209	80
N_{20}	167	47	214	80
N_{30}	178	48	226	80
LSD (5%)	33	9	37	2
Variation		*F- test values*		
I	0.0255*	0.0039**	0.0085**	0.0431*
N	0.470	0.0367*	0.1955	0.9999
I× N	0.837	0.1854	0.6004	0.7221
Contrasts				
I_0 vs $I_{1.5}$,I_3& $I_{4.5}$	0.0059**	0.0017**	0.0022**	0.0662
$I_{1.5}$ vs I_3& $I_{4.5}$	0.2285	0.0112**	0.0861	0.0925
I_3 vs $I_{4.5}$	0.4981	0.1557	0.3414	0.0456*
N_0 vs N_{10},N_{20}& N_{30}	0.2098	0.0060**	0.0619	0.9874
N_{10} vs N_{20}&N_{30}	0.5315	0.4096	0.4342	0.9613
N_{20} vs N_{30}	0.4683	0.6784	0.4445	0.9650

*and ** significant at 5 and 1% probability level, respectively
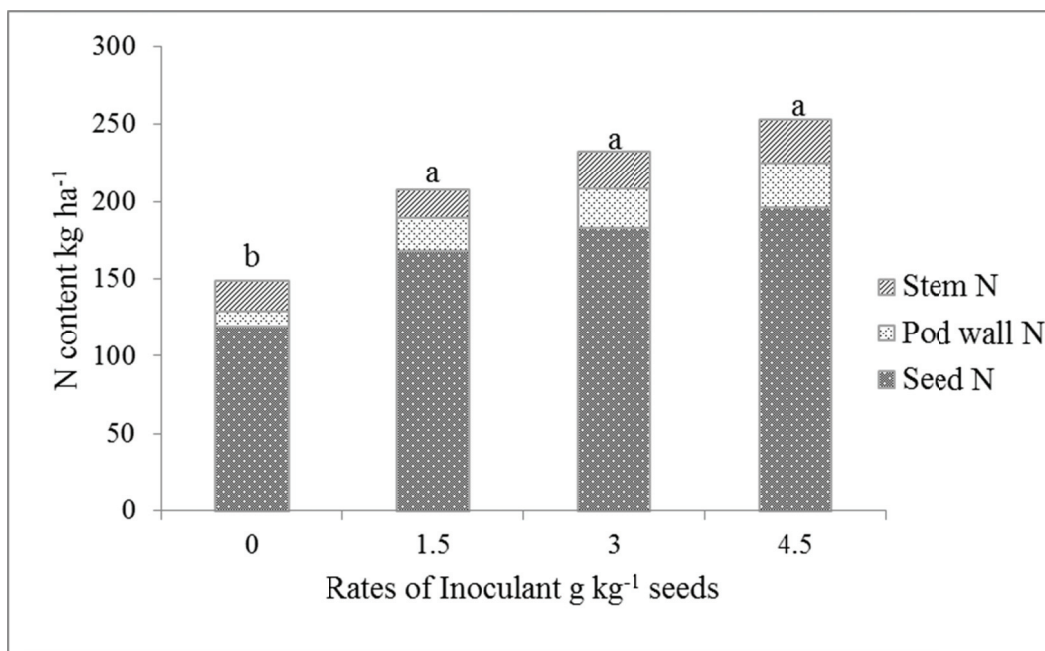
Figure 3.4 Total plants N distribution within the seed, pod walls, and stems of soybean plant with response to rates of inoculant. Means with same letter are not significantly different ($P < 0.05$).

The seeds accumulated a mean average of 80% of total plant N, while the pod walls and stem contained 11% and 10% of total plant N, respectively (Figure 3.4). Out of total plant N, the percentage of N accumulated in the seed and stem did not vary among the inoculated and the control plants but the percentage of N in the pod walls was comparatively higher in inoculated plants than in un-inoculated plants.

The seed N content was significantly higher in inoculated plants (Figure 3.5) while the seed N content of N fertilized plants did not vary among N0, N10 and N20. However, the seed N level of N30 treatment was similar to the lower rate of inoculant (IR1.5).
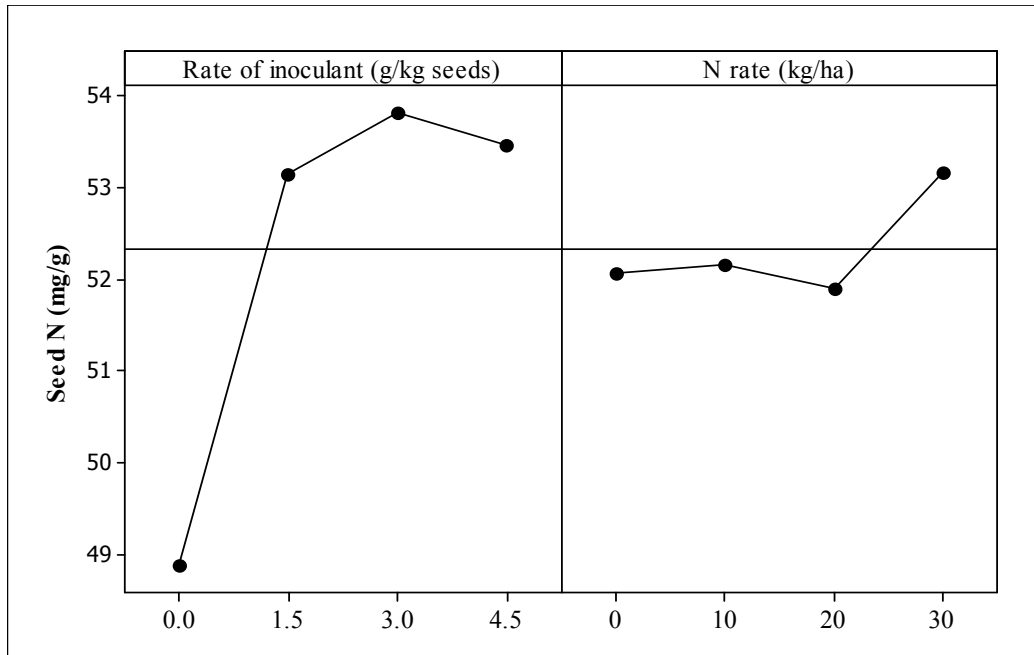
Figure 3.5 Seed N content with response to rates of inoculant at harvest. Means with same letter are not significantly different ($P < 0.05$).

3.4.4.2 Effects of *Bradyrhizobium* Inoculant and Starter N Rates on Soybean Leaf, Petioles, Stem, and Pod NO$_3$-N and K Composition.

The rate of inoculant and the rate of N did not show any significant relationship to the plant leaf, petiole, stem and pod sap NO$_3$-N or K at 60 DAP (Table 3.7) and 105 DAP (Table 3.8). At 60 DAP, the leaf, petiole and stem sap NO$_3$-N concentrations varied among the inoculant treatments in a similar pattern (Figure 3.6). Control plants showed an increasing trend in leaf, petiole, and stem sap NO$_3$-N concentrations. Unlike the petioles, there was an increasing trend of sap NO$_3$-N concentrations, with increased rates of inoculant for the leaves and stem (Figure 3.7). The rates of inoculant (Figure 3.8) and N treatments (Figure 3.9) demonstrated no remarkable variations on plant sap composition at 105 DAP.

Table 3.7 Effects of inoculation and starter N rate on plant leaf, petiole and stem sap NO_3-N and K concentration at 60 DAP (*F* test value)

Means	Leaf		Petiole		Stem	
Source	NO_3^-	K	NO_3^-	K	NO_3^-	K
I	0.1364	0.7771	0.1534	0.5023	0.9315	0.5018
N	0.7101	0.9433	0.7244	0.1193	0.2506	0.3857
I× N	0.3690	0.4071	0.1234	0.2335	0.9559	0.4841
Contrasts						
I_0 vs $I_{1.5}$,I_3& $I_{4.5}$	0.5465	0.3441	0.0635	0.9928	0.2352	0.8077
$I_{1.5}$ vs I_3& $I_{4.5}$	0.4987	0.8632	0.6082	0.1699	0.3177	0.1152
I_3 vs $I_{4.5}$	0.6508	0.8755	0.1936	0.6632	0.4687	0.8362
N_0 vs N_{10},N_{20}& N_{30}	0.3043	0.9884	0.9719	0.1374	0.3556	0.5816
N_{10} vs N_{20}&N_{30}	0.0947	0.7730	0.2606	0.8831	0.0738	0.6194
N_{20} vs N_{30}	0.6565	0.6158	0.9894	0.0544	0.2168	0.0271

*and ** significant at 5 and 1% probability level, respectively

Table 3.8 Effects of inoculation and starter N rates on plant leaf, petiole, stem and pod sap NO_3-N and concentration at 105 DAP (*F* test value)

Means	Leaf		Petiole		Stem		POD	
Source	NO_3^-	K	NO_3^-	K	NO_3^-	K	NO_3^-	K
I	0.2113	0.0101*	0.1443	0.3130	0.0656	0.0656	0.5606	0.3299
N	0.9357	0.2996	0.7906	0.9382	0.7429	0.7429	0.2768	0.3419
I× N	0.9796	0.3237	0.5643	0.7589	0.3523	0.3523	0.6329	0.4335
Contrasts								
I_0 vs $I_{1.5}$,I_3& $I_{4.5}$	0.9927	0.0274*	0.0873	0.5379	0.0355	0.0355	0.4045	0.7989
$I_{1.5}$ vs I_3& $I_{4.5}$	0.0509	0.0042**	0.2693	0.1227	0.3061	0.3061	0.8260	0.1132
I_3 vs $I_{4.5}$	0.6954	0.4243	0.1837	0.3953	0.0965	0.0965	0.2821	0.4324
N_0 vs N_{10},N_{20}& N_{30}	0.9924	0.5923	0.6736	0.8075	0.5677	0.5677	0.1625	0.7624
N_{10} vs N_{20}&N_{30}	0.5789	0.0715	0.3879	0.8604	0.8289	0.8289	0.3526	0.3647
N_{20} vs N_{30}	0.7540	0.8681	0.8039	0.5524	0.3614	0.3614	0.2993	0.1226

*and ** significant at 5 and 1% probability level, respectively
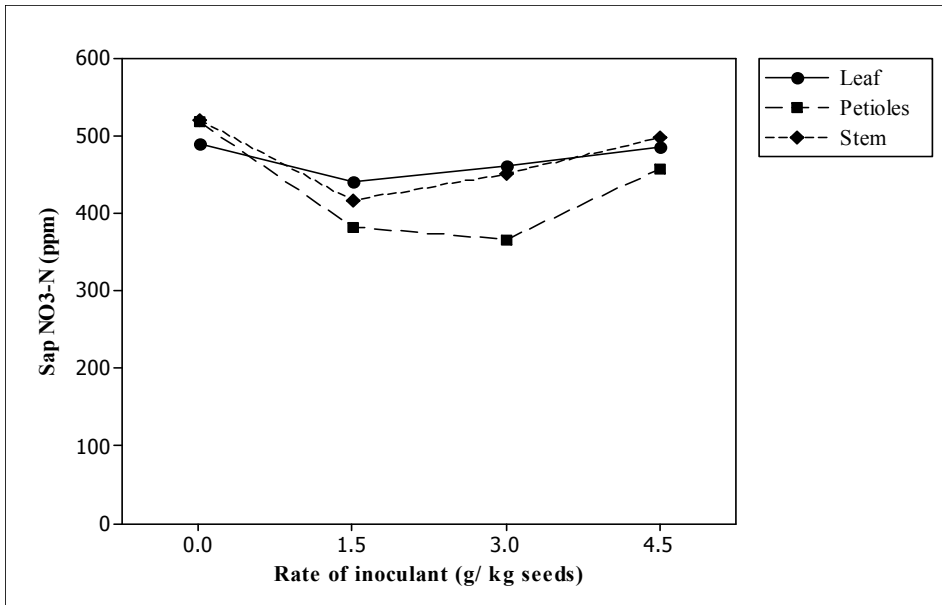
Figure 3.6 Soybean leaf, petioles, and stem sap NO$_3$-N concentration as affected by rates of inoculant at 60 DAP.
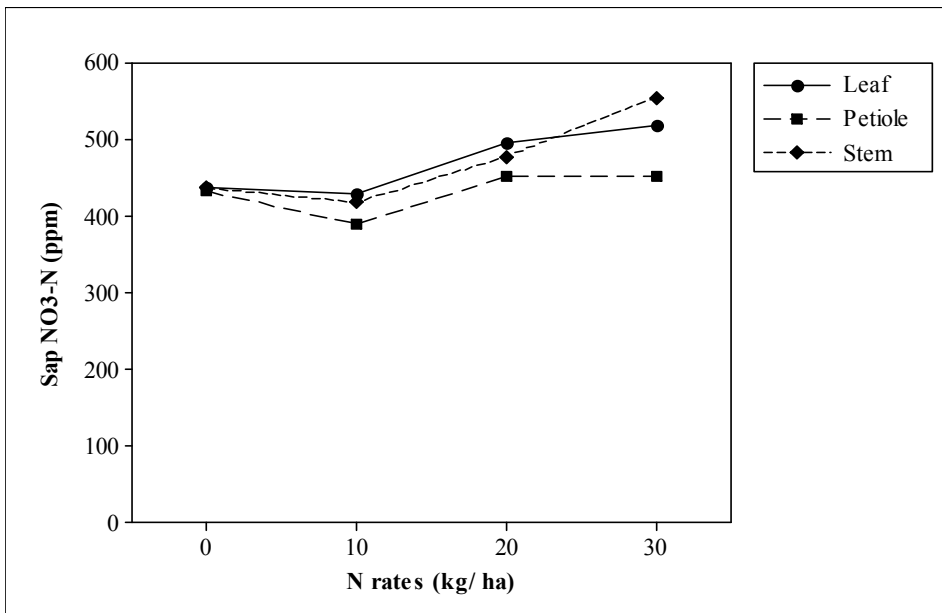
Figure 3.7 Soybean leaf, petioles, and stem sap NO$_3$-N concentration as affected by rates of N at 60 DAP.
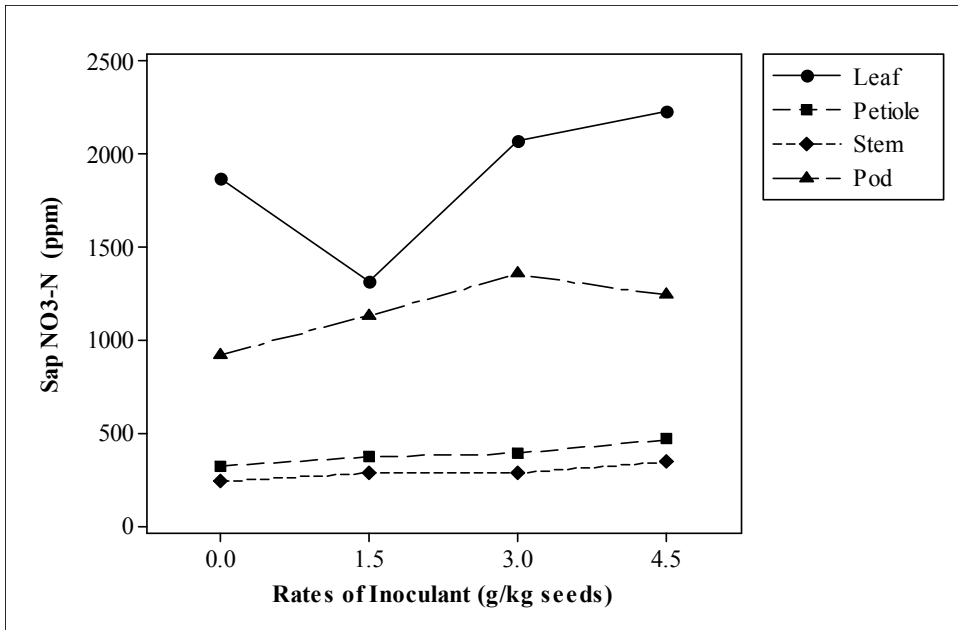
Figure 3.8 Soybean leaf, petioles, stem, and pod sap NO$_3$-N concentration as affected by rates of inoculant at 105 DAP.
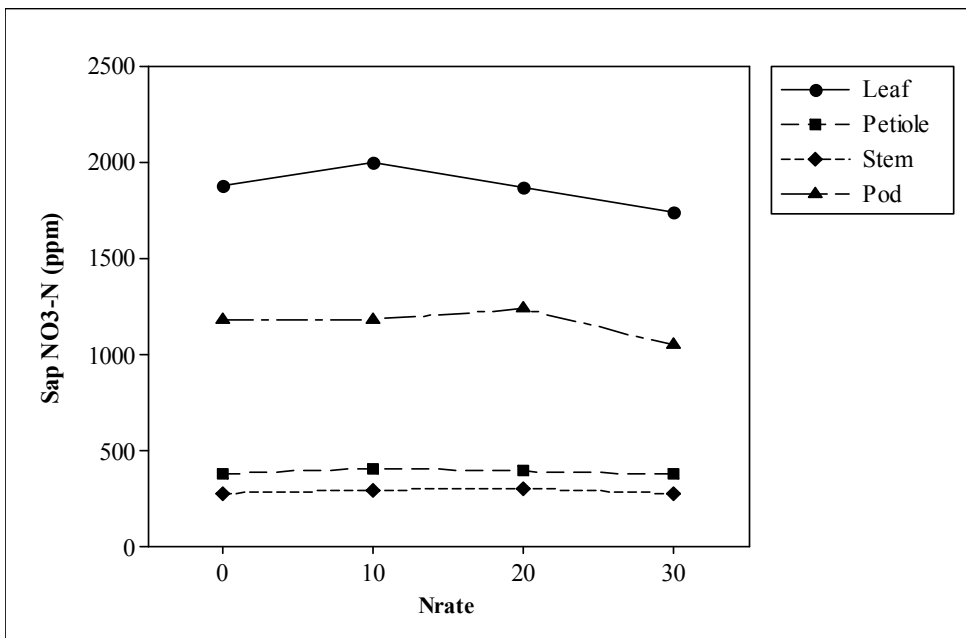
Figure 3.9 Soybean leaf, petioles, stem, and pod sap NO$_3$-N concentration as affected by rates of N at 105 DAP.

3.4.5 Effects of *Bradyrhizobium* Inoculant and Starter N Rates on Soybean Symbiotic N Fixation

The relative ureide percentage (RU%) was calculated by using the values obtained for stem ureide and NO_3-N analysis. The RU% was not significant at 60 DAP for either rates of inoculant and the starter N fertilizer. There was a significant increase in RU% *(P < 0.03)* for IR4.5 and IR3 at the seed filling stage (105 DAP) (Figure 3.10).
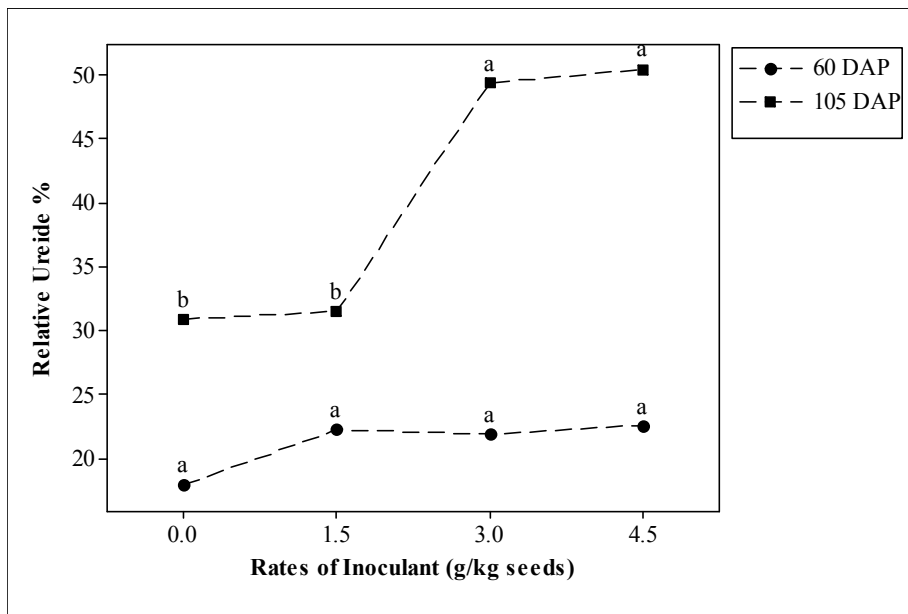
Figure 3.10 The relative ureide percentage at 60 and 105 DAP (LSD = 14 and LSD = 16 respectively; α =0.05) Means with same letters along a line are not significantly different *(P < 0.05)*.

The daily N fixation rates were calculated based on the RU% and the plant total N concentrations, obtained at different growth stages. At 60 DAP, there was no significant difference in the daily N fixation rate (Table 3.9) or in the cumulative N fixation from emergence to 60 DAP. Accordingly, the daily N fixation rate increased rapidly from 60 to 105 DAP. The daily N fixing activity increased in treatments IR4.5 and IR3, leading to higher cumulative N fixation from the early pod filling stage to the grain filling stage.

Table 3.9 Daily N fixation rate at 60 and 105 DAP and cumulative N fixation from emerging to 60 DAP and 60 DAP to 105 DAP as affected by inoculation and starter N rates at Wellington dykeland.

Means	Daily N fixation rate at 60 DAP kg ha^{-1}	Cumulative N fixation from emergence to 60 DAP kg ha^{-1}	Daily N fixation rate at 105 DAP kg ha^{-1}	Cumulative N fixation from 60 DAP to 105 DAP kg ha^{-1}
I_0	0.15	8.93	1.77	64.00
$I_{1.5}$	0.34	20.61	3.07	110.67
I_3	0.38	22.78	3.64	131.36
$I_{4.5}$	0.32	19.34	3.90	140.73
LSD (5%)	0.29	17.57	1.79	64.52
N_0	0.30	17.94	3.30	119.01
N_{10}	0.25	15.19	2.86	103.30
N_{20}	0.31	18.62	2.97	107.16
N_{30}	0.31	18.46	3.04	109.47
LSD (5%)	0.29	17.57	1.79	64.52
Variation		*F- test values*		
I	0.7775	0.7771	0.0463*	0.0463*
N	0.9441	0.9433	0.9771	0.9771
I× N	0.4080	0.4071	0.8309	0.8309
Contrasts				
I_0 vs $I_{1.5}$,I_3& $I_{4.5}$	0.3444	0.3441	0.0152*	0.0152*
$I_{1.5}$ vs I_3& $I_{4.5}$	0.8637	0.8632	0.3779	0.3779
I_3 vs $I_{4.5}$	0.8757	0.8755	0.7979	0.7979
N_0 vs N_{10},N_{20}& N_{30}	0.9855	0.9884	0.9827	0.9827
N_{10} vs N_{20}&N_{30}	0.7724	0.7730	0.8652	0.8652
N_{20} vs N_{30}	0.6190	0.6158	0.6797	0.6797

*and ** significant at 5 and 1% probability level, respectively

3.4.6 Variation in Soil Available N, pH, and EC with Respect to Rates of Inoculant and N Input

The soil available mineral NH_4^+ and NO_3^- were not significantly affected by N fertilizer application (Table 3.10). However, at 60 DAP, there was a limited increase in the soil NO_3^- levels with varying fertilizer N application rates (Figure 3.11). The soil pH and EC were stable over the research plot area at 60 DAP.

Table 3.10 Variation of the soil mineral N, EC, and pH with rates of inoculant and N fertilizer at 60 DAP

Means Source	Soil NH_4^+ (mg kg^{-1})	Soil NO_3^- (mg kg^{-1})	EC ds m^{-1} (1: 1)	pH (1: 2.5)	Soil moisture%
I_0	2.78	5.09	2.69	5.7	23
$I_{1.5}$	3.03	5.19	2.74	5.4	25
I_3	2.93	4.52	2.87	5.4	21
$I_{4.5}$	2.59	4.99	3.03	5.4	24
N_0	2.94	4.85	2.80	5.5	24
N_{10}	3.06	4.71	2.83	5.5	24
N_{20}	2.84	4.96	2.82	5.6	22
N_{30}	2.49	5.27	2.87	5.5	22
Variation		*F- test values*			
I	0.5190	0.4858	0.3293	0.3886	0.5060
N	0.2679	0.6139	0.6896	0.1807	0.3508
I× N	0.1216	0.4820	0.6076	0.5324	0.3283

*and ** significant at 5 and 1% probability level, respectively

3.4.7 Relationship between Soybean Nodule Number and Nodule Weight with the Rates of Inoculant and N Fertilizer

The rate of inoculant had a significant *(P < 0.0001)* linear effect on nodule weight; 36% variation was explained through the model (Figure 3.11). Similarly, there was a significant *(P < 0.01)* quadratic relationship between the rates of inoculant and the nodule number, accounting for 38% of the variation (Figure 3.12).
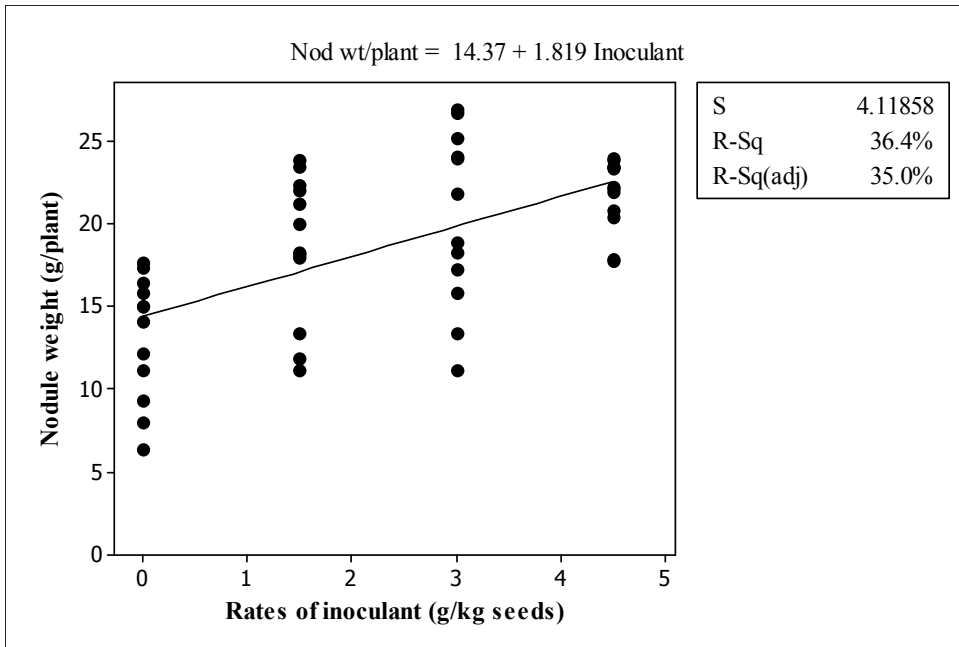
Figure 3.11 Relationship between applied rates of inoculant and soybean nodule weight at 60 DAP.
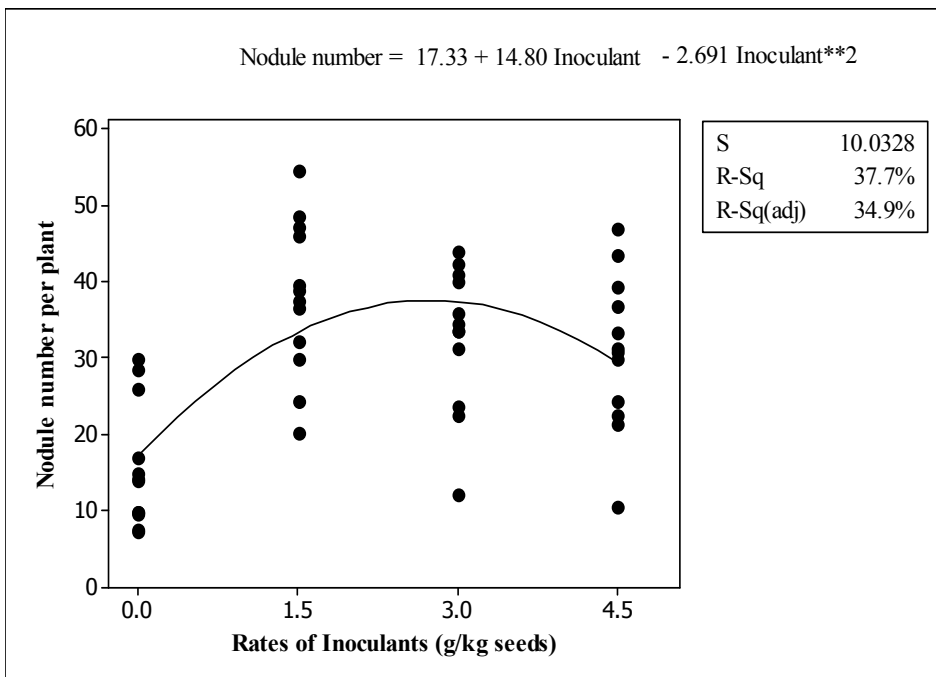
Figure 3.12 Relationship between applied rates of inoculant and soybean nodule number at 60 DAP.

3.4.8 Relationships between Soybean Yield, Total Biomass, Plant N with Rates of Inoculant and N Input

The rate of inoculant significantly influenced the soybean yield, seed N content, nodule numbers, and nodule weights. Regression analysis was carried out to establish the relationship between the different rates of inoculant and the soybean grain yield, TBM and seed N.
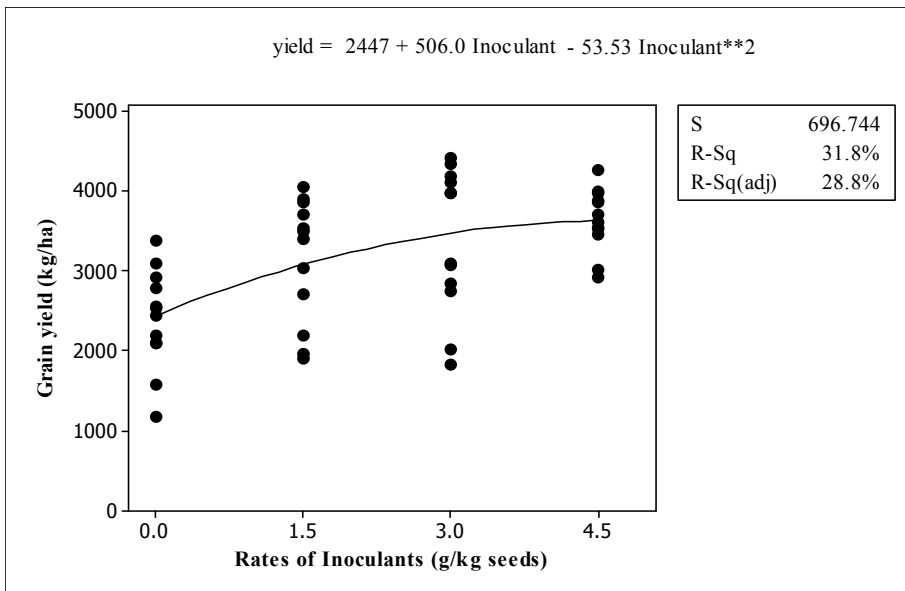
Figure 3.13 Relationship between applied rates of inoculant and soybean grain yield at harvest.

The grain yield showed a significant positive quadratic relationship with the rate of inoculant *(P < 0.0001)* that accounted for 32% variation (Figure 3.13). The linear model was only able to explain 29% of the variability that was accounted for by the rates of inoculant.

The rates of inoculant showed a quadratic effect on the soybean TBM at harvest *(P < 0.0001)* and the model explained 47% of the variation (Figure 3.14). The highest

TBM was achieved at IR3. The linear model was tested and 39.5% of total variation was explained by the model.

TBM = 712.2 + 253.9 Inoculant - 33.97 Inoculant**2

S	201.701
R-Sq	47.5%
R-Sq(adj)	45.2%
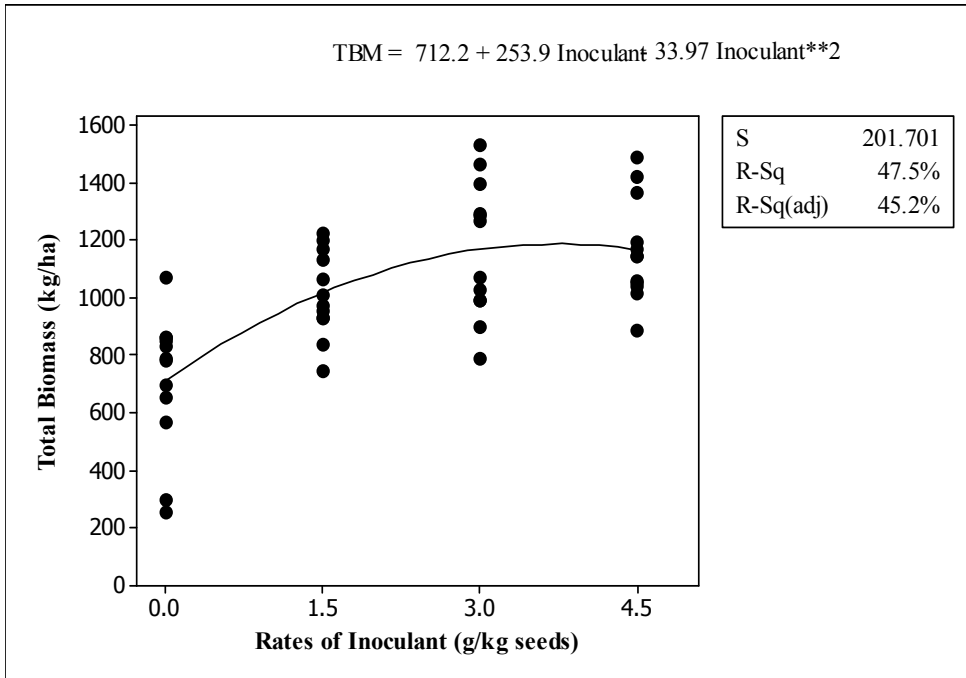
Figure 3.14 Relationship between applied rates of inoculant and soybean total biomass at harvest.

y = 261.8 + 13.80 total N uptake

S	247.931
R-Sq	91.2%
R-Sq(adj)	91.0%
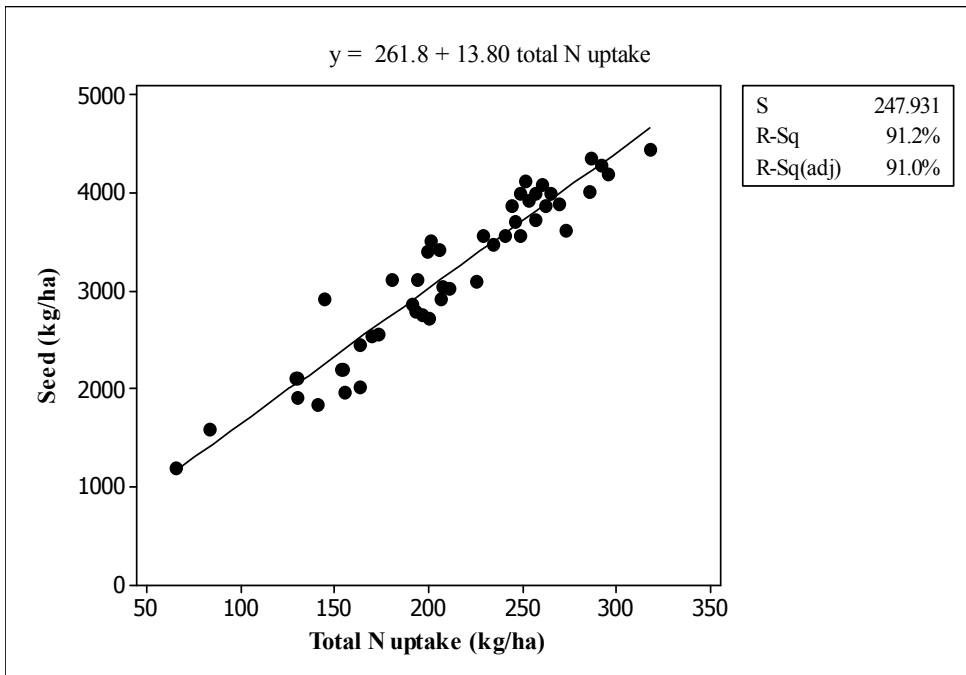
Figure 3.15 Relationship between total N uptake and the soybean grain yield at harvest.

There was a linear relationship between the total plant N uptake and the soybean yield. The effect was significant *(P < 0.0001)* and 91% variation was explained (Figure 3.15). The rate of the inoculant showed a significant *(P < 0.017)* quadratic effect on the soybean seed N content and 29% of the variation was accounted for through the model (Figure 3.16).

mg/g seed = 49.00 + 3.278 Inoculant - 0.5143 Inoculant**2

S	3.23813
R-Sq	28.7%
R-Sq(adj)	25.5%
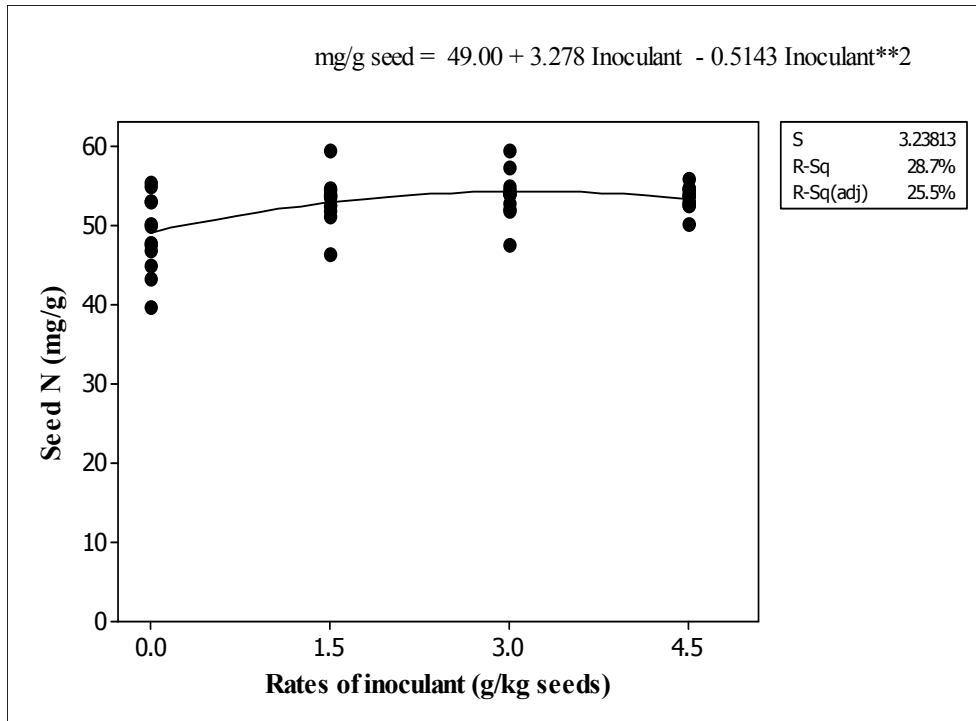
Figure 3.16 Relationship between total N uptake and the seed N content.

3.5 DISCUSSION

3.5.1 Soybean Nodulation

In this study, the nodule numbers and the nodule dry weights were significantly increased in all inoculated plants compared to un-inoculated plants. These signify that introduced *Rhizobia* and soybean performed better than un-inoculated plants on the dykelands. These results agree with the findings of Papakosta (1992) and Albareda et al (2009). The response to the inoculant can vary, depending on the *Rhizobial* population and the soil conditions. The number of nodules on the inoculated plants increased when there was less than 1×10^2 per gram of *Rhizobia* present in the soil (Singleton and Tavares, 1986). In our study, the nodule number increased significantly with the rate of inoculant over non-inoculated plants probably due to the poor existing *Rhizobial* population in the dykeland soil.

According to the Zengeni and Mpepereki (2003), the *Rhizobial* population size decreased with the increasing number of years since the last inoculation and survival rate varies with the soil type and conditions. This may be the reason that in our study, the un-inoculated plants were observed to produce significantly fewer nodules than inoculated plants. Since the field was subjected to crop rotation (corn-grass- soybean), the soybean had not been grown on the field for 2 years. The low survival rate of previously introduced *Rhizobia* and the lack of indigenous *Rhizobial* strains in our experimental field could be the reason that significantly higher numbers of nodules and higher nodule weight was observed in inoculated plants compared to un-inoculated plants. Albareda et al (2009) supported the necessity of soybean inoculation 3 years after last cultivation, and observed that the *Rhizobial* survival rate is highly dependent on the soil type and the

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bacterial strain whereas moderate acidic pH soil with sandy loam texture had lower survival capacity over the alkaline pH soil. Brockwell et al (1987) reported that there was a substantial reduction in the *Rhizobial* population of inoculum immediately after sowing until seed germination and the *Rhizobial* survival pattern was different, depending on the strain and the soil type (Brockwell et al., 1987). Accordingly, no difference in the nodule number among the rates of inoculant was observed in this present study. Although herbicides were used in the field, Moorman (1986) has reported that application of herbicides at a standard rate is not sufficient to reduce the *Rhizobial* population and soybean nodulation.

The low rates of inoculant IR1.5 and IR3 produced the maximum response for nodulation at N20 while high nodule formation was observed for higher inoculation rate (IR4.5) at N10. However, N fertilizer response on nodule number was not remarkable when compared to the individual inoculant levels without N fertilizer.

In agreement with Hungria et al (2006), a slight decrease in soybean nodule number was observed in this present study for the inoculation rates of IR1.5 and IR3 with N30. Starter fertilizer N application increases the soil available N and plants tend to uptake N from the soil to fulfill their N requirements at the vegetative stage. The soil available N may be sufficient to replenish plant N requirement, which inhibits the nodule formation. Tahir et al (2009) also reported a similar observation. Not only supplemented by N fertilizer, but also the higher available soil N and the mineralization rates in the field may be sufficient for plant growth at the vegetative stage. Therefore, the nodule formation by the existing *Rhizobia* can be suppressed due to the greater amount of soil mineral N. The soybean N demand is determined by the ability of the indigenous

Rhizobial population to compensate for the crop N requirement. However, our results indicated an inadequate and ineffective *Rhizobial* population in the dykeland soil.

3.5.2 Soybean Plant Growth and Yield

In the study, soybean seed yield obtained with the standard inoculant rate (3.4 kg t^{-1}) is in agreement with Bootsma et al (2005). According to the Maritime variety trials of Nova Scotia Crop Development Institute in 2010, the soybean cultivar 'Lynx' produced average of 3615 kg ha^{-1} of grain yield.

In the current, study PDW did not significantly increase with either the rate of inoculant or the nodule number at 60 DAP. In a previous study, Papakosta (1992) reported that the dry weight per plant is not influenced by nodulation in the early stages (32 and 68 DAP) of development. A significant increase of the PFW, PDW, PN, POFW, and PODW were observed in the inoculated plants over the control plants only at 105 DAP. At early vegetative stage, the soybean plant N requirement is less than at the reproductive stage. Therefore, available soil mineral N alone can be sufficient to fulfill plant N demand. As a result, plants did not show any variation at the early growth stage compared to the grain filling stage (105 DAP). Herridge (1982) suggested that soybean plants depend upon the mineral N for early growth stage and completely depends on the fixed N during the late reproductive growth. At the reproductive stage, the soybean plant N assimilation rate was high and soil mineral N alone was not adequate for the plant. Greater numbers of nodules on the inoculated plants facilitated atmospheric N fixation and ultimately increased the plant growth and yield. In the present study, at 105 DAP (R5 stage), the amount of total N derived from N fixation was greater than at 60 DAP (R2 stage) in the inoculated plants.

In the current study, grain yield and the RBM were significantly higher in inoculated plants than in the un-inoculated plants. An increasing trend in grain yield was observed from standard inoculant rate (3 g kg^{-1} seeds) to 150% of standard inoculant rate (4.5 g kg^{-1} seeds). The maximum RBM was observed in IR3 and IR4.5. Adequate N supply to the plant through N fixation and soil mineral N uptake could increase the plant biomass, resulting in higher grain yield and residual biomass. Several findings reported that inoculation with *Rhizobial* strains promotes higher soybean grain yield than in the un-inoculated plants (Egamberdiyeva et al., 2004; Diaz et al., 2009; Albareda et al., 2009). The increase in the grain yield was proportional to the increasing rate of applied *Rhizobia* (Papakosta, 1992). Greater nodule weight and the daily N fixing rate in inoculated plants increased N assimilation and led to higher grain yield and the residual biomass compared to the un-inoculated plants.

There was no significant effect of the applied starter N fertilizer on PFW, PDW, PN, PDW, grain yield and RBM. However, the N treatments produced a grain yield and total biomass similar to the lower rate (IR1.5) of inoculant. This lack of significant response to the fertilizer N application in this study agrees with the findings of several other researchers. Either application of a small amount of N fertilizer (Seneviratne et al., 2000; Hungria et al., 2006) or larger amounts of N fertilizer (200 kg ha^{-1}, 280 kg ha^{-1}) (Welch et al., 1973; Hungria et al., 2006; Diaz et al., 2009; Albareda et al., 2009) did not improve soybean yield. The residual biomass N was significantly high in fertilized plants compared to the non-fertilized plants and total biomass of N fertilized plants were not significantly different from the non-fertilized plants. However, total biomass N showed an increasing trend with the increasing N fertilizer rates. Diaz et al (2009) also reported

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an increase of plant dry matter with N fertilizer application. These results agree with the findings of Starlin et al (1998).

The different response, compared to previous studies, may be due to the soil and climatic factors. The amount of N fertilizer needed to achieve maximum seed yield response is larger for surface applied N than deep placement of controlled release N (Salvagiotti et al., 2009). This could be a reason that no significant positive response of starter fertilizer N application on the plant biomass was observed. Also, the field was supplemented with solid dairy manure every year prior to the planting and the crop residues from the previous year crop (corn) were tilled and introduced to the soil. A considerable mineralization occurred in the soil, possibly reducing the response to applied fertilizer N rates.

3.5.3 Soybean N Use Efficiency

As the field was subjected to the solid dairy manure application along with previous crop residues, the soil N mineralization could be high. Because of that, even with fertilizer N supply, the tissue N did not vary among the treatments at 60 DAP. Sinclair et al (2003) reported that at vegetative stage, plant N demand is preferentially accomplished by the soil mineral N and N fixation is initiated when the soil available N level is unable to meet the plant N requirement. At flowering stage (105 DAP), plant N demand usually is usually not accomplished through only the soil mineral N and fixed N then plays a key role in fulfilling soybean N requirement (Harper, 1974).

Corresponding to the rates of inoculant, the daily N fixation rate rose. This could be the reason for the increased tissue N concentration of inoculated plants at 105 DAP. Soybean N demand is relatively high in the later growth stages (Sinclair and DeWitt,

1976). However, the sap NO_3-N concentration showed an increasing trend with the rates of inoculant and the starter N fertilizer level at 60 DAP. However, there was no difference in sap NO_3-N at 105 DAP. Waterer (1997) reported that potato petiole sap NO_3-N levels reflected the rate and the time of fertilizer N application by changing with the increasing N fertilizer rates and time after planting.

The only factor that governed the seed N concentration was inoculation whereas, the 150% of standard inoculant rate did not influence an increasing in seed N content. Greater harvested plant total biomass N and seed N was found in inoculated treatments. The N fertilizer treatments showed similar seed N, TBMN and RBMN levels with the lowest inoculation rate (IR1.5). The adequate N supply during plant growth through N fixation and N uptake enhanced the plant biomass, resulting in an increase in the total N ha^{-1} in this field experiment. Also, many researchers have found that the inoculation can increase the plant N accumulation and grain N but the fertilizer N application cannot improve the N acquisition of soybean plant (Egamberadiyeva et al., 2004; Diaz et al., 2009; Albareda et al., 2009).

3.5.4 Soybean N Fixation

The relative ureide values obtained were according to the values observed by Osborne and Riedell (2006). However, the relative values reported by Osborne and Riedell (2011) were greater than values of the current experiment. This may be due to the reduction in symbiotic N fixation with high available soil N levels in the field. Relative ureide percentage did not vary in all treatments at 60 DAP. This may be due to the assimilation of NH_4^+-N in the soil. Herridge (1982) described the high ureide concentration in the shoot axis and the root through assimilation of NH_4^+-N by the plant

at the early growth stage. Symbiotic N fixation performance was greater with higher inoculant levels (IR3 and IR4.5) at 105 DAP. The soybean plant completely relies on fixed N at the late reproductive growth stage (Herridge, 1982). The response of the symbiotic N fixation is inconsistent with the rates of N fertilizer used for this study. Surface application of fertilizer N can suppress the BNF as fertilizer N can support the plant N requirement (Salvagiotti et al., 2009). The inoculant rates IR3 and IR4.5, without N fertilizer, may be sufficient to fulfill total N demand. With higher levels of inoculant, the plant nodulation increased and as a result, the daily N fixation rates increased. In this study, inoculant rate IR3 and IR4.5 had the greater number of nodules, leading to higher N fixation rates.

3.6 CONCLUSION

The results described here indicate that inoculation of soybean can facilitate the plant growth and the grain yield in the dykelands. It is necessary to inoculate the soybean if the field is subjected to three-year crop rotation in moderately acidic pH soils with a loamy texture. There were no significant differences in the grain yield of 50%, 100% and 150% of standard inoculant rate. However, application of 4.5 g kg^{-1} seeds of inoculant showed an increasing trend in soybean symbiotic N fixation in the acidic dykeland soil conditions, with a yield increasing trend. The application of N fertilizer at N30 suppressed soybean nodulation. At the vegetative stage, the N nutrient requirement of the soybean plant can be fulfilled by acquisition of soil mineral N, if the soil is rich in mineral N. Thus, application of solid dairy manure and incorporation of previous year crop residues to the soil increased the soil available mineral N pool through

mineralization. Application of starter fertilizer at low rates (10, 20, and 30 kg ha^{-1}) could not increase the soybean grain yield. However, starter N showed a rising trend in the plant total biomass at harvest. At the early stage of the soybean plant growth, the N requirement is comparatively low. If the soil mineral N levels and symbiotic N fixation together can fulfill the soybean N demand at the early vegetative stage, there is no benefit in applying starter N fertilizer on the dykelands.

CHAPTER 4   **EFFECTS OF USE OF *BRADYRHIZOBIUM* INOCULANT WITH STARTER N FERTILIZER ON SOYBEAN SYMBIOTIC N$_2$ FIXATION AND PLANT GROWTH IN SALINE DYKELAND SOIL CONDITIONS**

4.1. INTRODUCTION

High yielding soybean plants have a greater N nutrient demand, which is fulfilled by mineral N uptake and symbiotic N fixation (Harper, 1974). The efficiency of symbiotic N fixation is governed by soil available N, compatibility of symbiotic partners and yield limiting factors (Keyser and Li, 1992). In some studies, there is a good correlation between the rates of inoculant and the soybean seed yield (Papakosta, 1992; Albareda et al., 2009).

Several reserches have focused on the effect of N fertilizer on soybean-rhizobium symbiosis and N fixation where N fertilizer is applied to soybean at the early growth stage or at the reproductive stage. Application of N fertilizer significantly increased the soybean grain yield and plant growth, when the symbiotic N fixation alone could not achieve the plant N demand (Thies et al., 1995). Sometimes, soybean responses to N fertilizer is inconsistent (Barker and Sawyer, 2005; Gan et al., 2003). Several factors can limit the soybean symbiotic N fixation under field conditions. Increasing soil salinity reduced the symbiotic N fixation by affecting the initial steps of the nodule formation (Zahran, 1999). Moisture stress at the early vegetative stage has been found to reduce soybean nodulation and symbiotic N fixation (Pena-Cabriales and Castellanos, 1993).

Dykeland soils are formed by tidal action. These soils are subjected to rapid moisture fluctuations, together with high salinity (Beke, 1990).

4.2 OBJECTIVES

The objective of this study was to investigate soybean symbiotic N fixation responses with respect to *Bradyrhizobium japonicum* inoculation and starter N fertilizer on a saline dykeland site.

4.3 MATERIAL AND METHODS

4.3.1 Site Description

The experiment was conducted during the summer of 2010 in the Habitant dykeland located in the Annapolis Valley in Nova Scotia (NS). The soil type is an Acadia marine loam (silty clay loam in texture). According to the previous year (fall 2009) soil analysis, the soil (depth of 0-15 cm) chemical properties were 8.4 mg NH_4^+, 19.4 mg NO_3^-, 80 mg P, and 200 mg K per kilogram of soil nutrients with pH of 6 and 5.2 ds m^{-1} of EC. The field was subjected to 3-year crop rotation. In the first year was soybean followed by corn in the second year, followed by grass in the third year (corn-grass-soybean). The field was cultivated with corn the previous year.

4.3.2 Field Experimental Setup

The treatments were arranged in a split plot design with three replications where main plots were arranged in a RCBD. The treatments consisted of four rates of inoculant as well as starter N fertilizer. The inoculant rates were 0 (IR0), 1.5 (IR1.5), 3 (IR3-recommended rate of 'Nitragin' commercial inoculant) and 4.5 (IR4.5) g kg^{-1} seeds and the starter N fertilizer rates were 0 (N0), 10 (N10), 20 (N20) and 30 (N30) kg N ha^{-1}. The seeding was carried out on June 10, 2010 using soybean cultivar "Lynx RR". The rates of inoculant were used as the main plot treatments while the starter N rates as the sub plot

treatment. The reason to select the rates of inoculant as the main plot factor was to minimize the cross contamination. Different rates of inoculant were randomly assigned to the four main plots in each block and the N fertilizer treatments were randomly assigned to the subplots within each main plot (Figure 4.1). The plot size was 2 m in width and 4 m in length (area of 8 m^2). Inter and intra row spacing was 17.5 cm and 9.5 cm, respectively. There was a 0.5 m buffer zone in between 2 sub-plots and 48 subplots randomly arranged within three blocks.

Block/Rep 1

IR_0					$IR_{4.5}$					IR_3					$IR_{1.5}$			
N_0	$N2_0$	N_{10}	N_{30}		N_{30}	N_{20}	N_{10}	N_0		N_0	N_{20}	N_{30}	N_{10}		N_{20}	N_{10}	N_{30}	N_0

Block/Rep 2

$IR_{1.5}$					IR_3					$IR_{4.5}$					IR_0			
N_0	N_{30}	N_{10}	N_{20}		N_{30}	N_{10}	N_{20}	N_0		N_{10}	N_0	N_{20}	N_{30}		N_{10}	N_{20}	N_0	N_{30}

Block/Rep 3

IR_0					IR_3					$IR_{1.5}$					$IR_{4.5}$			
N_{10}	N_0	N_{20}	N_{30}		N_0	N_{20}	N_{30}	N_{10}		N_{30}	N_{20}	N_{10}	N_0		N_0	$N1_0$	N_{20}	N_{30}
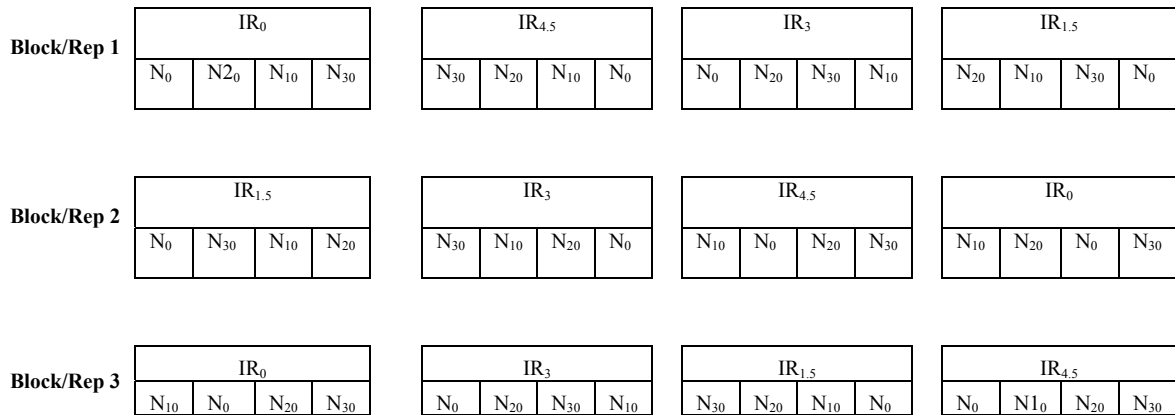
Figure 4.1 Field experiment layout in Habitant dykeland

The commercial inoculant "Nitragin" (peat mixture) was used to inoculate soybean seeds. For the inoculation, the seed sowing, fertilizer applications, and weeding, the methods described in the Chapter III section 3.2.2 were followed.

4.3.3 Soybean Field Plant Sampling

The plant samples were obtained as described in Chapter III section 3.2.3. The sampling stages were R2 (Full flower/60 DAP) and R5 (begin seed/90 DAP). The harvest samples could not obtain at R7 (physiological harvest maturity/132 DAP) due to an unavoidable circumstance. Uneven distribution of plant stands were observed because of

poor seed germination. Therefore, from each plot, 10 plants were randomly selected at R2 and R5 stages. Plants above ground biomass were divided into leaves, petioles, stem, pods, and nodulated root for the analysis of ureide-N and NO_3-N. The dried stem parts were ground using the Wiley mill and passed through the 60 mesh size (1 mm).

The plant height, root length, nodule numbers, nodule weights, PON, POFW and PFW were determined. The samples were oven dried at 60 ^{o}C to a constant weight to quantify the total N content. The total plant dry weights (PDW) and pod dry weights of oven dried samples were measured. Dried plant parts were ground and passed through the 60 mesh size (1 mm) screen.

The collection of soybean plant leaf, petiole, stem, and pod (R2 and R5) saps and determination of the sap NO_3-N and K were conducted according to the methods described in Chapter III section 3.2.3.

4.3.4 Soil Sampling

The soil samples were obtained at 60 DAP to measure the effects of the applied N fertilizer on soil mineral N level as described in Chapter III section 3.2.4. The sub samples from each plot pooled together and representative soil samples were obtained. The samples were air dried and the debris was removed. Later the dried samples were sieved through 2 mm mesh size. Processed samples were stored for chemical analysis.

4.3.5 Soybean Plant Chemical Analysis

The plant chemical components measured were plant total N and stem ureide to quantify the N uptake and the symbiotic N fixation respectively as described in the Chapter III section 3.2.5.

4.3.5.1 Plant Total N

The plant total N was determined according the method described in Chapter III section 3.2.5.1 (Bremner and Mulvaney, 1982). The total plant N was calculated for g plant^{-1}.

4.3.5.2 Quantification of Stem Ureide Compounds

The ground stem tissue samples were used to extract the ureide compounds (Herridge, 1982), and 0.5 g of sample was taken into a boiling tube and boiled with 25 ml of distilled water for 1-2 minutes in a boiling water bath. The filtration was done through a Whatman No: 40 filter paper into a 50 ml volumetric flask. The residues were washed into the filter and rinsed with the distilled water. When the content was cooled, the filtrate was volumerized up to 50 ml with distilled water. The concentration of ureide N was measured by the Young and Conway method (1942) and NO_3-N by the Cataldo method (Cataldo et al., 1974) as described in Chapter III (section 3.2.5.2).

Calculation of daily N fixing activity and daily N absorption rate

The following equation was used to calculate the relative abundance of ureide similar to the Chapter III section 3.2.5.2,

% of N derived from atmospheric N_2 (RU%) = $\dfrac{4\times \text{Ureide- N}}{[4\times\text{Ureide- N} + \text{Nitrate- N}]} \times 100$

N fixation and absorption rates were estimated according to the method described by the Tewari et al., (2004). Daily N fixing activity and the daily N absorption rates were calculated as mentioned in Chapter III section 3.2.5.2.

4.3.6 Soil Chemical Analysis

Soil available N, soil pH (1: 2.5, water), and EC (1: 1) were measured using the methods described in Chapter III section 3.2.6.

4.3.7 Statistical Analysis

Before running the ANOVA, the normality and constant variance were checked by using Minitab 15 statistical software. The independence was assumed to be through randomization. The PROC MIXED procedure was used in SAS 9.2 statistical software for the data analysis. The block and the interaction between the block and inoculant were considered as the random effects. The rate of inoculant and the rate of starter N were considered as fixed effects. The statistical significant criteria was a Type III error rate of $P = 0.05$ with 95% confidence interval. When the interaction effects were significant, as the multiple mean comparison method LS means (Least Square Means) was used. If the main treatment effects (rate of inoculant and rate of starter N) were significant, LSD (Least Square Difference) analysis was computed to evaluate the difference in means. Orthogonal contrasts were constructed for the inoculant and N fertilizer comparison. For, all the measured variables the contrasts were performed by comparing the different levels of inoculants and starter N rates.

4.4 RESULTS

4.4.1 Effects of *Bradyrhizobium* Inoculant and Starter N Rates on Soybean Plant at Vegetative Stage

The emergence of seedlings was observed at 15 DAP. Even though planting was carried out prior to the rain, the Habitant site did not get adequate moisture required for seed germination. After sowing, there was one week of drought period, resulting in poor seed germination (65%) throughout the field. The plants flowered around 50 DAP and fertilized plants produced flowers earlier than the inoculated plants. The pod elongation started around 60 DAP. Moisture level in the field changed rapidly throughout the experiment. For example, on certain days, the field was water logged, while on other days the soil was totally dried and cracked.

PFW, PDW and plant heights did not show statistically significant responses, either for interaction or main effects by rate of inoculant and rate of fertilizer at 60 DAP (Table 4.1). There were no significant differences among the N treatments in terms of PFW, PDW and plant height.

The increasing rate of inoculant significantly reduced the root length. Therefore, the highest root length was observed in IR0 plants and the lowest root length was found in IR4.5. Although there was no significant difference in the root length of N fertilized plants, the root length of all N rates were larger than the higher rate of inoculant (IR4.5) (Table 4.1).

Table 4.1 Plant fresh weight (PFW), dry weight (PDW), plant height, root length, nodule number and nodule weight as affected by inoculation and starter N rates at the Habitant dykeland at 60 DAP

Means Source	PFW(g)	PDW(g)	Plant height (cm)	Root length (cm)	Nodule number	Nodule fresh weight (g)
I_0	15	3.99	25	12	6	0.09
$I_{1.5}$	14	3.94	24	12	11	0.60
I_3	15	4.12	25	11	14	0.54
$I_{4.5}$	14	3.70	25	10	19	0.88
LSD (5%)	2	1.08	6	1.29	8	0.29
N_0	14	3.63	25	11	12	0.50
N_{10}	16	3.94	25	11	8	0.48
N_{20}	14	4.24	25	11	13	0.50
N_{30}	14	3.93	24	12	17	0.62
LSD (5%)	2	1.08	6	1.29	8	0.29
Variation			_F- test values_			
I	0.9672	0.8045	0.8686	0.0251*	0.0219*	0.0076**
N	0.1255	0.5572	0.7023	0.8550	0.1983	0.7400
I× N	0.2616	0.3050	0.6893	0.3820	0.1066	0.2644
Contrasts						
I_0 vs $I_{1.5}, I_3$ & $I_{4.5}$	0.6935	0.8577	0.7507	0.0196*	0.0133*	0.0022**
$I_{1.5}$ vs I_3 & $I_{4.5}$	0.8973	0.9403	0.5779	0.1056	0.0989	0.4019
I_3 vs $I_{4.5}$	0.8177	0.3677	0.6348	0.1832	0.2639	0.0497*
N_0 vs N_{10}, N_{20} & N_{30}	0.2282	0.2436	0.4983	0.9237	0.7830	0.7915
N_{10} vs N_{20} & N_{30}	0.0384	0.6972	0.3405	0.5581	0.0568	0.5031
N_{20} vs N_{30}	0.9975	0.4707	0.9301	0.5242	0.3355	0.4021

*and ** significant at 5 and 1% probability level, respectively.

The interaction between the rates of inoculant and the rates of N fertilizer application did not significantly affect the nodule numbers. The nodule numbers and the nodule weights of the inoculated plants significantly increased, compared to the un-inoculated plants. The higher inoculant rate (IR4.5) produced a higher nodule weight compared to other treatments (Table 4.1).

4.4.2 Effects of *Bradyrhizobium* Inoculant and Starter N on Soybean Plant at Seed Filling Stage

The plants were at the seed filling stage at 105 DAP. The PFW, PDW, PN, POFW and PODW were not significantly affected by the interaction effect of the rates of the inoculant and the rates of N fertilizer, nor did the other factors influence the response to the treatments. The rate of N did not increase the PFW, PDW, PN, POFW, and PODW (Table 4.2).

Table 4.2 Soybean plant fresh weight, dry weight, pod number, pod fresh weight, and pod dry weight as affected by inoculation and starter N rates at the Habitant dykeland at 105 DAP

Means Source	Plant Fresh weight(g)	Plant Dry weight (g)	Pod Number	Pod Fresh weight (g)	Pod Dry weight (g)
I_0	87	10	35	45	14
$I_{1.5}$	101	13	43	59	15
I_3	93	11	41	55	15
$I_{4.5}$	93	11	36	51	14
LSD (5%)	22	12	10	15	4
N_0	85	11	37	48	12
N_{10}	97	11	39	54	16
N_{20}	99	12	42	55	16
N_{30}	94	11	40	53	14
LSD (5%)	22	12	10	15	4
Variation			*F- test values*		
I	0.6211	0.7353	0.4559	0.3311	0.8589
N	0.5346	0.6867	0.8257	0.7765	0.2176
I× N	0.9150	0.7180	0.8612	0.9895	0.9479
Contrasts					
I_0 vs $I_{1.5}$,I_3& $I_{4.5}$	0.3423	0.6154	0.1880	0.1355	0.4832
$I_{1.5}$ vs I_3& $I_{4.5}$	0.3972	0.3552	0.5171	0.3595	0.8790
I_3 vs $I_{4.5}$	0.9791	0.9024	0.5978	0.6379	0.6983
N_0 vs N_{10},N_{20}& N_{30}	0.1671	0.7428	0.4518	0.3189	0.0635
N_{10} vs N_{20}&N_{30}	0.9972	0.6046	0.6916	0.9404	0.6937
N_{20} vs N_{30}	0.6494	0.3149	0.7008	0.8038	0.3714

*and ** significant at 5 and 1% probability level, respectively.

4.4.3 N Uptake of Soybean Plant at Different Growth Stages

4.4.3.1 Effects of Rates of Inoculant and Starter N Soybean Plant Total N

The plant tissue N content did not significantly increase with the rates of the inoculant and the rates of N at 60 and 105 DAP (Table 4.3). Although, the plant total N content increased in IR1.5 treatment compared to IR0, IR3 and IR4.5 at 105 DAP, the effect was not significant.

Table 4.3 Soybean total plant N as affected by inoculation and starter N rates at 60 and 105 DAP at the Habitant dykeland.

Means Source	Plant N at 60 DAP g plant^{-1}	Plant N at 105 DAP g plant^{-1}
I_0	0.090	0.254
$I_{1.5}$	0.098	0.332
I_3	0.096	0.250
$I_{4.5}$	0.100	0.261
LSD (5%)	0.029	0.095
N_0	0.085	0.270
N_{10}	0.093	0.266
N_{20}	0.102	0.302
N_{30}	0.104	0.260
LSD (5%)	0.029	0.095
Variation	*F- test values*	
I	0.7751	0.2688
N	0.4181	0.8089
I× N	0.2481	0.8474

*and ** significant at 5 and 1% probability level, respectively.

4.4.3.2 Effects of Inoculant and Starter N Rates on Soybean Leaf, Petioles, Stem and Pod sap NO$_3$-N and K Composition.

The plant leaf and stem sap NO$_3$-N and K compositions were not significantly elevated by the rates of the inoculant and the rates of N fertilizer at 60 DAP (Table 4.5). Among the different treatments, the variability of the NO$_3$-N and K was inconsistent. However, there was a significant interaction between the rates of inoculant and N rates

for sap NO_3-N at 105 DAP. Also, the interaction effect of the rates of inoculant and N fertilizer rates was significant on leaf and pod sap K concentrations (Table 4.6). The higher levels of leaf (Figure 4.2) and pod (Figure 4.5) sap NO_3-N were observed for the IR1.5-N20 treatment combination. The following treatment combinations also had comparatively higher levels of leaf sap NO_3-N: IR0-N30, IR4.5-N20, IR1.5-N30 and IR3-N0. The NO_3-N contents of stem (Figure 4.4) and petiole saps (Figure 4.3) were significantly high in the IR3-N0 treatment. Similar to the leaf sap, higher pod sap NO_3-N was found in IR0-N30, IR4.5-N20, and IR1.5-N20 treatment combinations. The interaction effect of the rates of inoculant and the fertilizer N were inconsistent for both leaf and pod K concentrations.

Table 4.4 Effects of inoculation and starter N rate on plant leaf, petiole, and stem sap NO_3-N and K concentration at 60 DAP (*F* test value)

Means	Leaf		Petiole		Stem	
	NO_3^-	K	NO_3^-	K	NO_3^-	K
I	0.1651	0.1362	0.1534	0.5023	0.6413	0.1860
N	0.5007	0.0911	0.7244	0.1193	0.5290	0.9221
I× N	0.5025	0.2543	0.1234	0.2335	0.4343	0.1500
Contrasts						
I_0 vs $I_{1.5}$,I_3& $I_{4.5}$	0.6177	0.5600	0.0635	0.9928	0.7536	0.2204
$I_{1.5}$ vs I_3& $I_{4.5}$	0.0930	0.0360*	0.6082	0.1699	0.3791	0.0744
I_3 vs $I_{4.5}$	0.1299	0.4975	0.1936	0.6632	0.4081	0.9611
N_0 vs N_{10},N_{20}& N_{30}	0.8349	0.2020	0.9719	0.1374	0.6137	0.8933
N_{10} vs N_{20}&N_{30}	0.1583	0.2189	0.2606	0.8831	0.1711	0.5097
N_{20} vs N_{30}	0.5903	0.0403	0.9894	0.0544	0.9279	0.9474

*and ** significant at 5 and 1% probability level, respectively.

Table 4.5 Effects of inoculation and starter N rates on plant leaf, petiole, stem, and pod sap NO_3-N and K concentration at 105 DAP (*F* test value)

Means	Leaf		Petiole		Stem		POD	
	NO_3^-	K	NO_3^-	K	NO_3^-	K	NO_3^-	K
I	0.7115	0.3080	0.0301*	0.7242	0.0086**	0.6177	0.0196*	0.3683
N	0.0204*	0.9968	0.0037**	0.8127	0.0008**	0.2497	0.0080**	0.3155
I× N	0.0028**	0.0276*	0.0001**	0.5665	<0.0001**	0.1989	0.0009**	0.0213*

*and ** significant at 5 and 1% probability level, respectively.
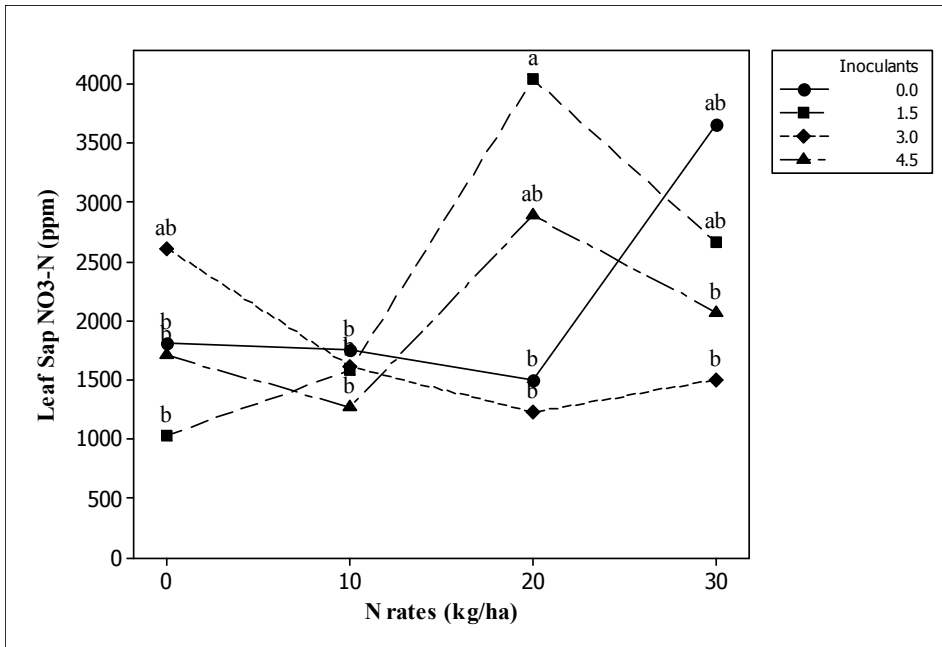
Figure 4.2 Soybean leaf sap NO₃-N concentrations as affected interaction effect of inoculation and N rates at 105 DAP. Means with same letters are not significantly different *(P < 0.05)*.
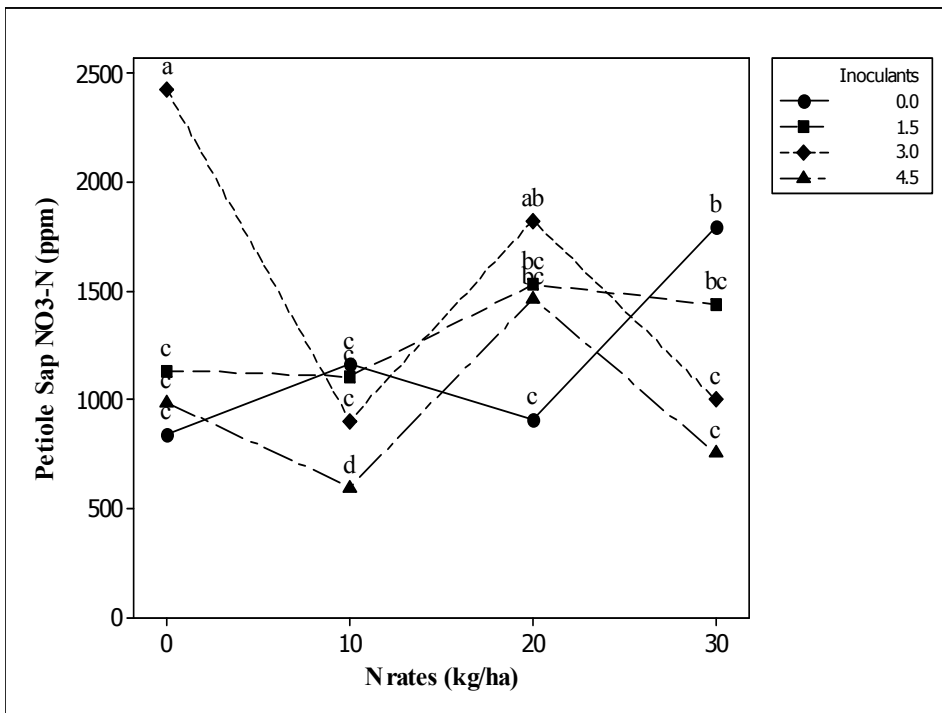
Figure 4.3 Soybean petiole sap NO₃-N concentrations as affected by interaction effect of inoculation and N rates at 105 DAP. Means with same letters are not significantly different *(P < 0.05)*.
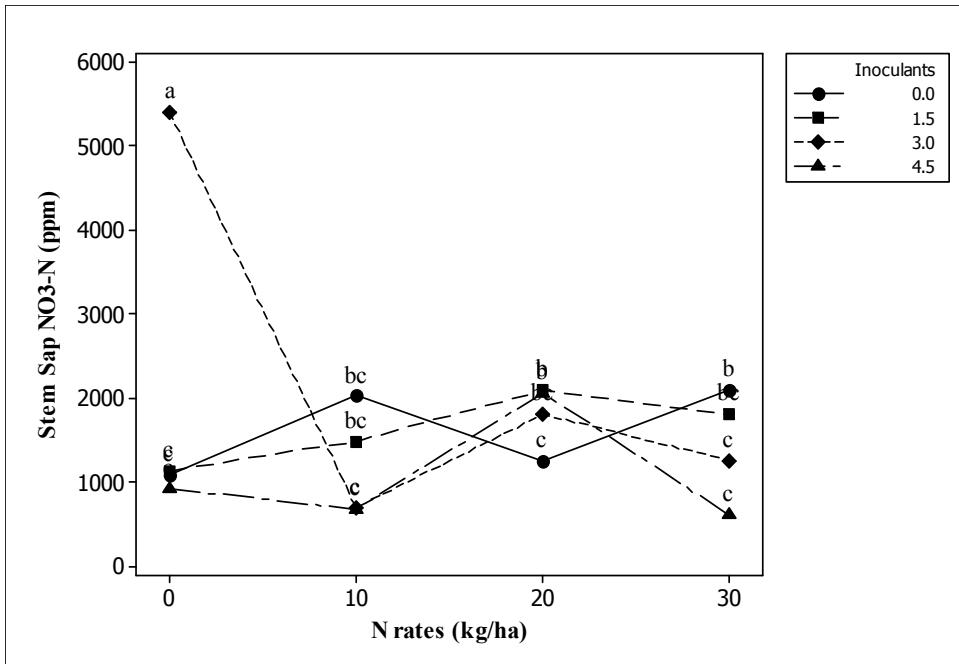
Figure 4.4 Soybean stem sap NO$_3$-N concentrations as affected by interaction effect of inoculation and N rates at 105 DAP. Means with same letters are not significantly different *(P < 0.05)*.
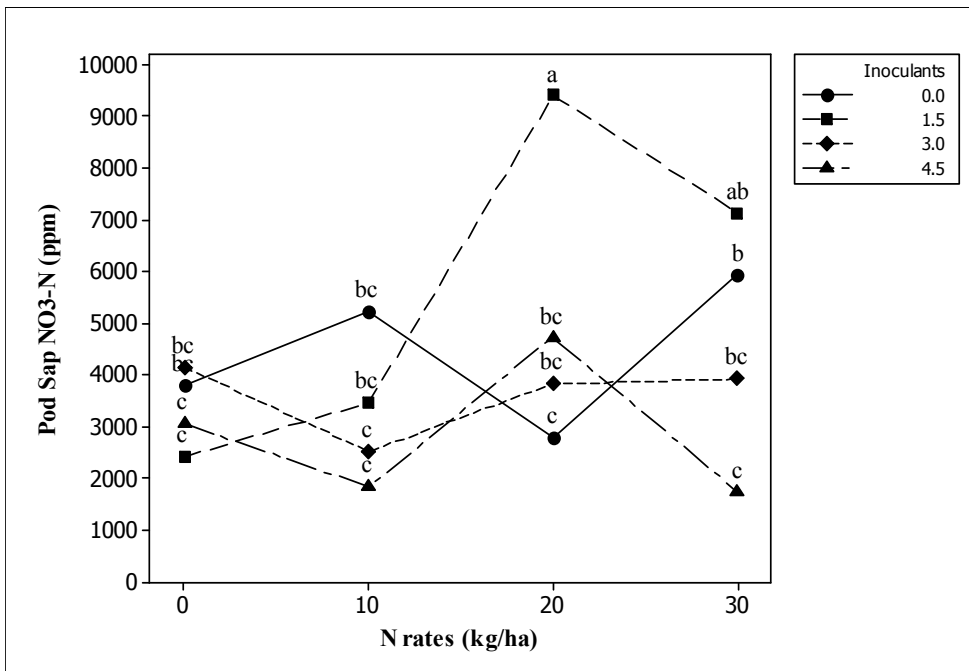
Figure 4.5 Soybean pod sap NO$_3$-N concentrations as affected by interaction effect of inoculation and N rates at 105 DAP. Means with same letters are not significantly different *(P < 0.05)*.

4.4.4 Effects of Rates of Inoculant and Starter N on Soybean Symbiotic N Fixation.

The RU% was calculated, based on the stem ureide and NO_3^--N concentrations. Ther of inoculant had no significant effect on RU%. Surprisingly, the stem RU% showed a declining trend with increasing rates of inoculant. Maximum RU% was observed with un-inoculated plants at 60 and 105 DAP (Figure 4.6). There was a decreasing trend in RU%, with an increasing inoculant rate. The daily N fixation rates and the daily N absorption rates were determined, based on the plant total N content. The daily N fixation rates were not significantly affected by either rates of inoculant nor rates of starter fertilizer at 60 or 105 DAP (Table 4.7).
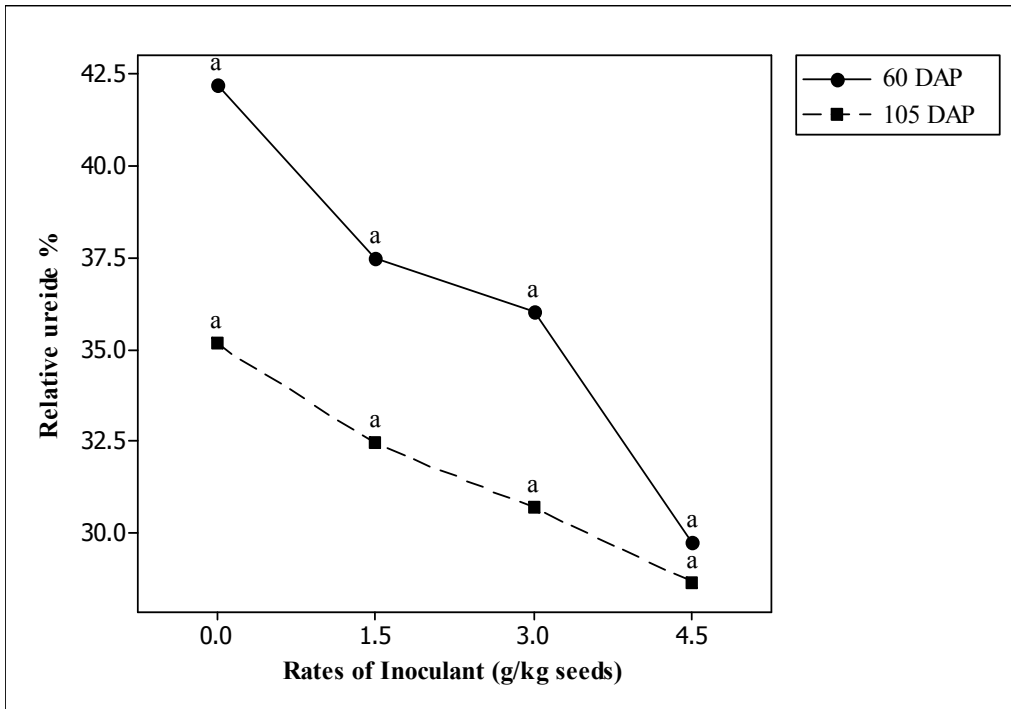
Figure 4.6 The relative ureide percentage at 60 and 105 DAP. Means with same letters along a line are not significantly different *(P < 0.05)*.

Table 4.6 Daily N fixation rates and daily N absorption rates as affected by inoculation and starter N rates at 60 and 105 DAP at Habitant.

Means	Daily N fixation rate at 60 DAP	Daily N fixation rate at 105 DAP	Daily N absorption rate at 60 DAP	Daily N absorption rate at 105 DAP
Source	kg ha^{-1}D^{-1}	kg ha^{-1}D^{-1}	kg ha^{-1}D^{-1}	kg ha^{-1}D^{-1}
I_0	0.26	0.96	0.36	0.97
$I_{1.5}$	0.29	0.86	0.43	0.88
I_3	0.26	0.94	0.50	0.94
$I_{4.5}$	0.26	0.98	0.61	0.99
N_0	0.25	1.00	0.36	1.10
N_{10}	0.25	1.03	0.58	1.09
N_{20}	0.30	0.80	0.42	0.67
N_{30}	0.25	0.90	0.54	0.91
Variation		*F- test values*		
I	0.9529	0.7997	0.1987	0.9713
N	0.8952	0.3503	0.1300	0.2684
I× N	0.2618	0.8554	0.8186	0.9329

*and ** significant at 5 and 1% probability level, respectively.

At the early growth stage (60 DAP), the daily N absorption rates were increased in both inoculated and N fertilized plants without any significant effects and they were higher than daily N fixation rates. However, the daily N absorption rate increased rapidly at 105 DAP and did not vary with either the rates of inoculant or rates of N (Table 4.7).

4.4.5 Relations between Soybean Nodulation with Rates of Inoculant and N at 60 DAP.

The nodule numbers and the nodule weights were significantly affected by the rates of inoculant, as mentioned above. Regression analysis was conducted in order to evaluate the relationship between applied rates of inoculant on soybean nodulation. Inoculant rates had a significant positive linear effect on soybean nodule numbers *(P < 0.014)* that accounted for 17.2% of variation (Figure 4.7).

Interestingly, there was a significant linear positive relationship between the soybean nodule weights *(P < 0.0001)* and the rates of inoculant that accounted for 33% of variation (Figure 4.8). The quadratic model also illustrated how the effect of applied rates of inoculant on nodule weights accounted for 34% of total variation.
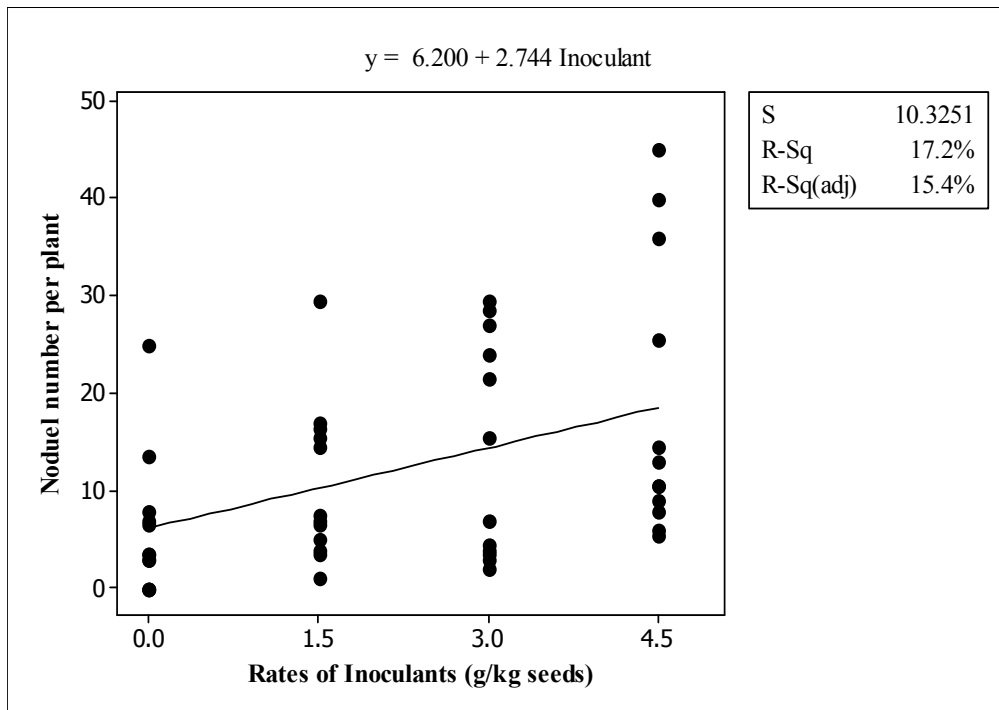
Figure 4.7 Relationship between applied rates of inoculant and nodule number at 60 DAP.
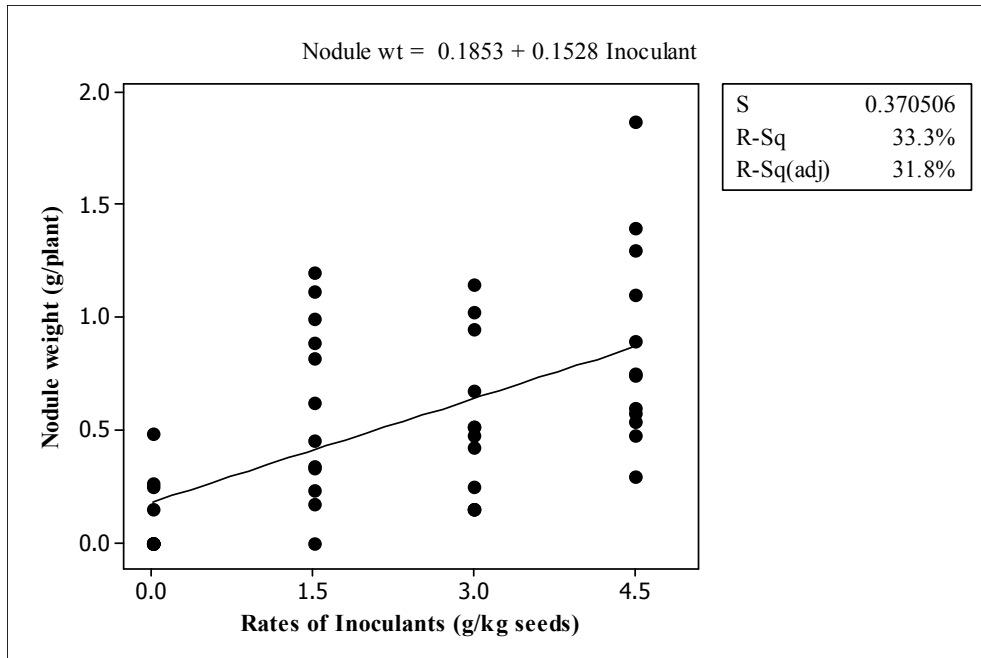
Figure 4.8 Relationship between applied rates of inoculant and nodule weight at 60 DAP.

4.4.6 Relationship between Soybean Plant Biomass and Plant N Uptake

The regression analysis revealed that there was a significant linear relationship between the plant dry matter content and total N uptake at both sampling stages *(P < 0.0001)*. The N uptake accounted for 79% (Figure 4.9) and 87% (Figure 4.10) of the variability at 60 and 105 DAP, respectively. Moreover, the PN showed a significant linear relationship with plant N uptake (Figure 4.11). Analysis did not revealed any better relationship (linear, quadratic or cubic) between applied rates of inoculant with PFW, PN, and plant total N.
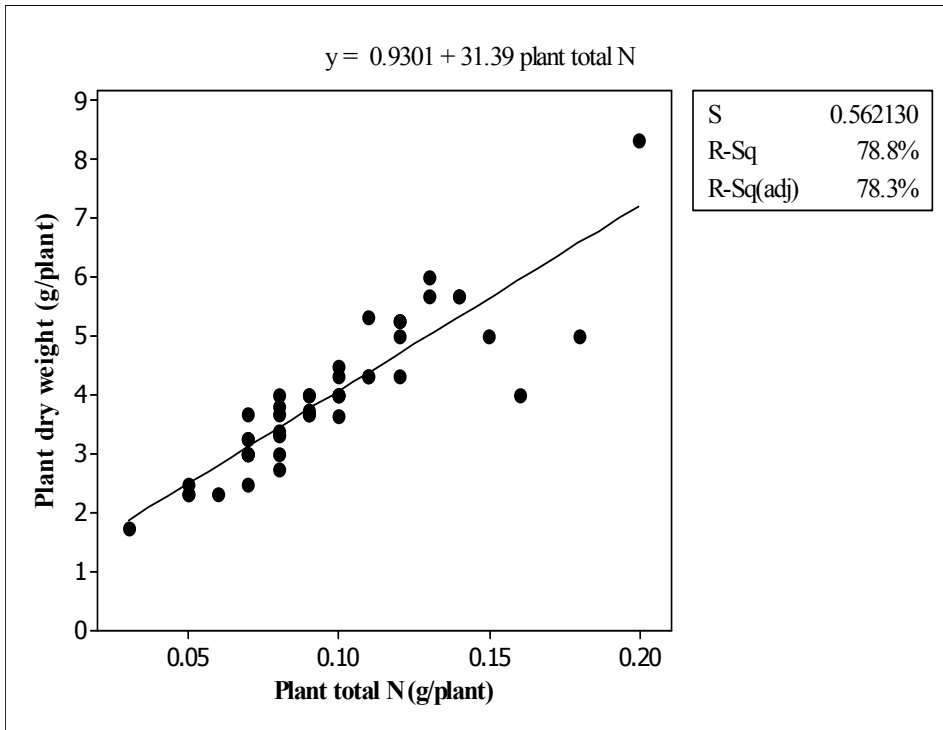
Figure 4.9 Relationship between total N uptake and the plant dry weight at 60 DAP.
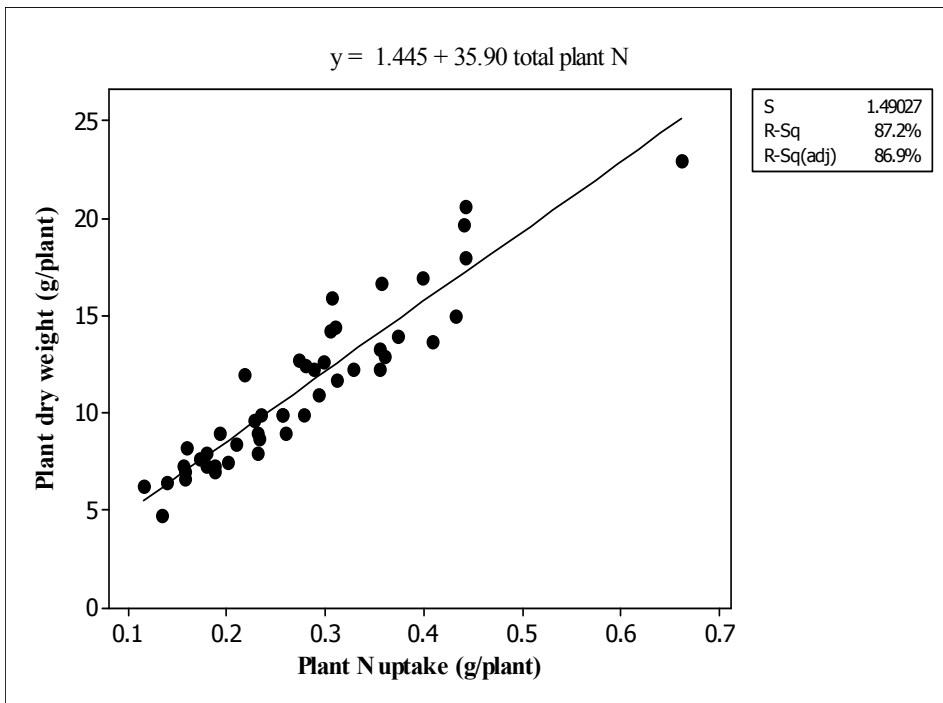
Figure 4.10 Relationship between total N uptake and the plant dry weight at 105 DAP.
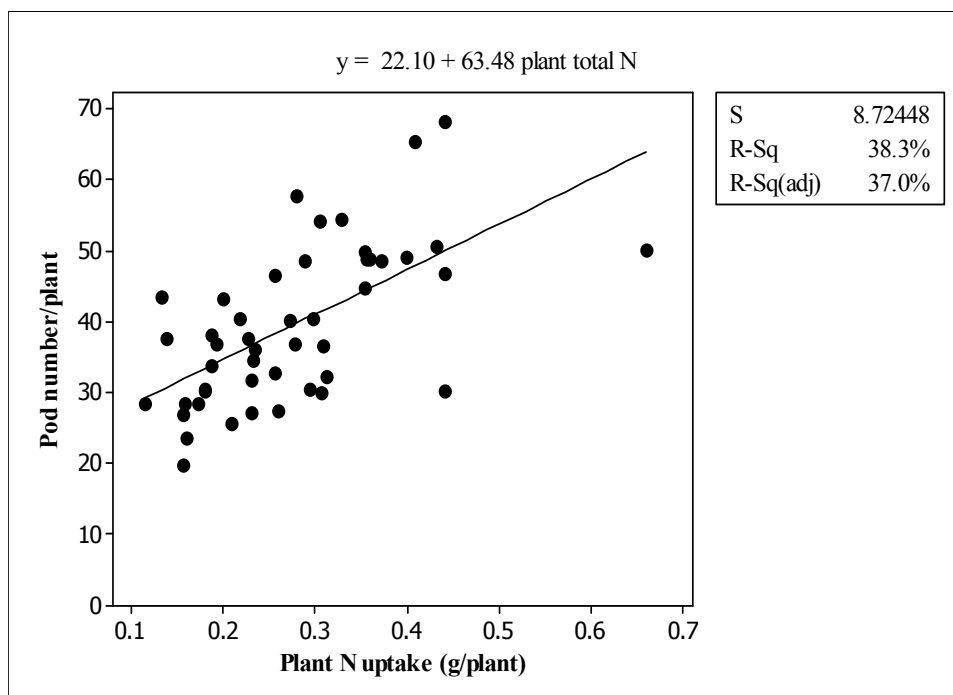
Figure 4.11 Relationship between total N uptake and the pod number at 105 DAP.

4.4.7 Variation in Soil Available N, pH and EC with Respect to Rates of Inoculant and N Input

There was no significant variation in soil pH, EC and available mineral N in the experimental site (Table 4.7). Soil available mineral NH_4^+ and NO_3^- did not effect by the rates of N fertilizer. The soil EC levels were greater than 4 ds m^{-1}, indicating soil salinity in the field. The soil pH was stable over the research plot area. The higher soil pH values were due to the presence of Na^+ and Ca^{2+} ions.

The soil N did not correlate with following parameters: plant total N and PFW (Table 4.8). However, the plant total N showed a significantly *(P < 0.016)* negative correlation with soil EC. The nodule numbers and the nodule weights were negatively correlated with soil NO_3^-. Also, the nodule numbers were negatively correlated with soil EC. There was a significant quadratic *(P < 0.021)* relationship between soil EC and plant total N content at 60 DAP (Figure 4.12). The model accounted for 18% of the variation.

Table 4.7 Variation of the soil mineral N, EC and pH at 60 DAP

Means Source	Soil NH_4^+ (mg/ kg)	Soil NO_3^- (mg/ kg)	EC ds m^{-1} (1:1)	pH (1:2.5)
I_0	2.33	3.73	4.7	6.8
$I_{1.5}$	2.10	3.78	5.6	7.0
I_3	2.14	3.11	4.5	6.9
$I_{4.5}$	2.20	3.48	5.0	6.9
N_0	2.21	3.61	4.5	6.9
N_{10}	1.99	3.81	5.9	6.9
N_{20}	2.35	3.32	4.7	6.9
N_{30}	2.21	3.36	4.8	7.0
Variation		*F- test values*		
I	0.8039	0.5922	0.3993	0.3886
N	0.5972	0.6050	0.1152	0.1807
I× N	0.5262	0.6179	0.1762	0.5324

*and ** significant at 5 and 1% probability level, respectively.

Table 4.8 Pearson Correlation values for soil mineral N concentration, plant total N and biomass at 60 DAP

Parameter	Rate of inoculant	N rate	Plant N	Plant biomass	Nodule number	Nodule weight
Soil NO_3-N	-0.199	-0.152	-0.005	-0.067	-0.188	-0.198
Soil NH_4-N	-0.041	0.059	-0.102	-0.029	0.012	-0.245
EC	0.029	-0.047	-0.382*	-0.266	0.025	-0.123

g/plant = 0.1814 - 0.000242 EC + 0.000000 EC**2

S	0.0289878
R-Sq	17.7%
R-Sq(adj)	13.1%
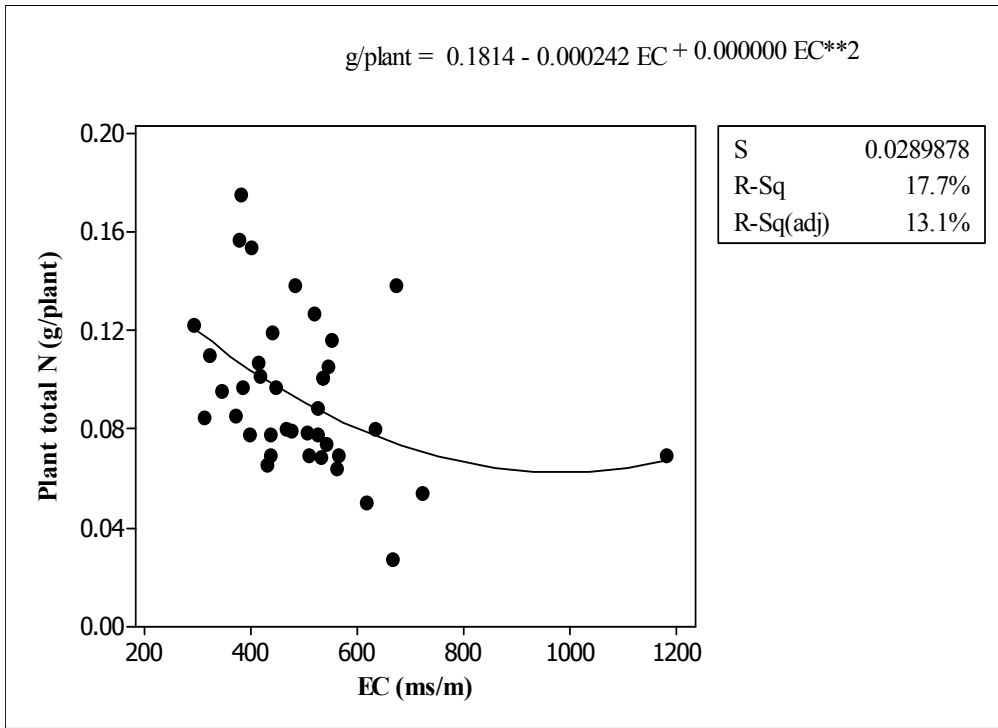
Figure 4.12 Relationship between total N uptake and the EC at 105 DAP.

4.5 DISCUSSION

4.5.1 Soybean Nodulation

The inoculation of soybean in the Habitant field significantly increased the nodule numbers and the nodule weights. According to the results, the nodule weights were not proportionate to the nodule numbers. The IR1.5 had significantly fewer nodules but the nodule weight was similar to that of IR4.5. In the Wellington field, the nodule numbers were comparatively higher than those in the Habitant field. This may be due to the combined effects of drought and saline soil conditions at sowing. These two factors reduced the survival rate of the introduced *Rhizobia,* subsequent infection, and the nodule formation. Current study demonstrated that the desiccation was one of the major factors, which governed the survival of inoculated *Rhizobia* on the seeds, and it is in agreement with the studies of Vincent et al., (1962), Griffith et al., (1992), and Zahran, (1999). Zahran (1999) explained that under the presence of moisture stress, *Rhizobia* tends to change their cell morphology and this eventually reduces root infection and nodulation of legumes.

In the experimental field, the soil EC levels ranged from 4.7 to 5.8 ds m^{-1}, resulting in drastic reductions in the nodule numbers and the nodule weights; this agrees with the research findings of Elsheikh and Wood, (1995) and Rao et al., (2002). The reduction of soybean nodulation due to high EC levels resulted from the distraction of signal molecule exchange (Singleton and Bohlool, 1984; Velagaleti and Marsh, 1989; Jayasundara et al., 1998), *Rhizobial* attachment (Howieson et al., 1993), root hair curling, and initiation of infection thread (Evans et al., 1980). In agreement with Serraj and

Sinclair (1996), this study also observed a few larger nodules with more smaller nodules because of soil salinity.

In the present study, the nodule numbers showed an increasing trend, with high N levels in the un-inoculated plants. The existing *Rhizobia* in the soil might have infected the root nodules of N20 and N30 treatments in response to high soil NH_4^+ levels. Current observations tally with previous research data of Gan et al., (2008), and Van Heerden et al., (2008). According to the findings of Sprent and Thomas (1984), the low and moderate levels of starter N fertilizer stimulated the formation and development of nodules when the seedlings consumed the N from the cotyledons and active N fixation was delayed. When soil *Rhizobia* was present in the field application of fertilizer N increased the soil NH_4^+ levels and promoted the soybean nodulation. Under saline conditions, the survival rate of the introduced *Rhizobial* strain could be low and thus delayed root infection and nodule formation.

4.5.2 Soybean Plant Growth

Due to the saline soil conditions, only 65% of seed germination was observed. Essa (2003) also, reported poor seed germination due to soil salinity. Several studies have shown that application of starter N fertilizer increased the soybean plant growth and the seed yield (Barker and Sawyer, 2005; Taylor et al., 2005; Gan et al., 2002; Osborne and Ridell, 2006) while, some studies reported contradictory findings (Welch et al., 1973; Duong et al., 1984). In the present study, significant positive responses were not observed, either with the rates of inoculant or the rates of N fertilizer on soybean plant biomass at both vegetative (60 DAP) and seed filling (105 DAP) stages. However, the

fertilized treatments without inoculant were able to produce the same amount of plant biomass as inoculated plants. This agrees with the findings of Baker and Sawyer (2005). Kubota et al (2008) reported that N fertilizers could offer short-term benefits in unfavourable years without negative effects in favourable years.

Studies have reported significant reductions of shoot and root dry weights due to soil salinity (Velagaleti and Marsh, 1989; Munns 1993; Cordovilla et al., 1995; Miransari and Smith, 2007; Miransari and Smith, 2009). Similarly, the Habitant field with saline soil conditions, showed a reduction in the plant biomass compared to non-saline Wellington (2.8 ds m^{-1}) field. Legume plants are more sensitive to salinity than plants totally depend on mineral N (Zurayk et al., 1998). In this study, the plant growth retardation could be due to the poor nutrient uptake by the plants because of excessive levels of NaCl and the toxic ions in the soil.

Root length decreased with the increasing rates of inoculant and rose with the higher rates of N. Surprisingly, plants with larger numbers of root nodules and the highest root length (IR4.5, IR1.5, IR3, and N30) exhibited greater levels of plant total N at 60 DAP. It has been reported that application of N fertilizer mitigates the negative effects of water stress by enhancing root growth for better soil exploration, where plants are able to get adequate water for growth and transportation of fixed N (Gan et al., 2008). Since the current field site was highly subjected to moisture stress at the early growth stage this could be the reason that N fertilized plants had elevated levels of tissue N without any significant effects. In the present study, the lack of response for applied fertilizer N agreed with the studies of Welch et al., (1973), Hungria et al., (2006), Diaz et al., (2009), and Albareda et al., (2009).

Although sap NO_3-N composition was significantly affected by the interaction of rates of inoculant and the rates of N fertilizer at 105 DAP, the plant total N content did not demonstrate evidence of interaction effect. Especially in saline conditions, it is disadvantageous to depend upon the soil mineral N content because of the superior competition for plant nutrient uptake. Moreover, the introduced *Rhizobial* strain was unable to fix N effectively under the saline soil conditions.

4.5.3 Symbiotic N Fixation

The soybean symbiotic N fixation is more sensitive to salinity than the plant's growth (Elsheikh and Wood, 1995). The research implied that there was no difference between the applied rates of inoculant and the un-inoculated plants for RU%, and daily N fixing rates. This may be due to the adverse influence of soil salinity on N fixation. It has been reported that chickpea formed nodules at a salinity level of 6 ds m^{-1}, where the N fixation was completely inhibited (Zurayk et al., 1998). The current study is consistent with the above observation, since the inoculated plants did not show any difference in daily N fixation rates compared to the un-inoculated plants in the presence of high number of nodules.

The effect of NaCl on nodule nitrogenase could be due to the reduction of the phloem sap supply to the nodules (Serraj et al., 1998). For instance, Serraj et al (1996b) reported that ureide could be accumulated in the nodule as a result of drought stress. Factors that decrease the phloem flow reduce the export of fixed N from the nodule that leads to accumulation of fixed N products in the nodules (Serraj et al., 1999a). The accumulation of ureide compounds can trigger the accumulation of intermediate

compounds in the plant (Serraj et al 1996b). In agreement with the above findings, the greater nodule numbers did not enhance the daily N fixation rate in the present study.

4.6 CONCLUSION

In conclusion, we reported that application of inoculant at a rate of 4.5 g kg^{-1} seed promoted the soybean nodule weight, compared to the standard rate of 3 g kg^{-1} seed on saline dykeland soil conditions. Moreover, the formation of the greater number of nodules did not enhance N fixation, where it was suppressed by soil salinity. N fixation was not evident on the dykelands, even with plants having large numbers of nodules. However, a salinity level of 4.5 ds m^{-1} in this dykeland soil suppressed soybean symbiotic N fixation. Not only N fixation, but also soybean plant growth was severely suppressed by the soil salinity. It is advantageous to use saline tolerant *Rhizobial* strains for soybean inoculation to mitigate deleterious effects of soil salinity.

CHAPTER 5   SOYBEAN SYMBIOTIC NITROGEN FIXATION RESPONSES AND GRAIN YIELD IN RELATION TO *BRADYRHIZOBIUM* INOCULATION AND STARTER NITROGEN USE UNDER CONTROLLED ENVIRONMENT CONDITIONS

5.1. INTRODUCTION

Soybean plant requires a large quantity of N for its growth. At the seed filling stage, the soybean plant N demand is high and a large portion of N in the vegetative tissues are translocated to the developing seeds, causing a rapid decline in the leaf N content that reduces the photosynthetic capacity (Sinclair and DeWitt, 1975). There is a greater dependency on photosynthesis by legume plants compared to the plants reliant on soil N and fertilizer N (Kaschuk et al., 2009). The cost of N assimilation is up to 2.5 g C g^{-1} N uptake, while the N fixation cost ranges between 5.2-18.8 g C g^{-1} N (Minchin and Witty, 2005). N fixation may be limited by photosynthesis rate and the availability of photosynthate (Imsande, 1988).

Increasing inoculant rate increased the soybean grain yield, plant total N and grain N (Papakosta, 1992). There are studies, which emphasized the benefits of fertilizer N application on soybean grain yield and protein content in the field. Also, it has been reported that the use of low rates (26.6 kg ha^{-1}) of starter N enhanced soybean grain yield (Morshed et al., 2008). The observations from the two field trials (chapter 3 and 4) were influenced by climatic and soil factors, such as moisture stress and soil salinity.

5.2 OBJECTIVES

In this study, the impact of different application rates of *Bradyrhizobium* inoculant and starter N fertilizer on soybean grain yield, nodulation, leaf chlorophyll

content, photosynthesis rates, and N fixation were investigated under controlled environment conditions.

5.3 MATERIALS AND METHODS

The growth chamber study was carried out similar treatments as the field experiments because there were some drawbacks in the field experiments in observing the soybean plant nodulation. There were four rates of inoculant, 0 (IR0), 1.5 (IR1.5), 3 (IR3- recommended rate) and 4.5 (IR4.5) g kg^{-1} seeds. "Nitragin" was used as the *Bradyrhizobium* inoculant. Based on results from the field experiments, N level applications for the growth chamber plants were calculated. In the field experiment, there were four rates of N 0, 10, 20 and 30 kg ha^{-1}. Based on field planting density, the amount of fertilizer N available to a soybean plant was calculated for different N rates. The starter N supply was determined to be 0, 20, 40, and 60 mg of N $plant^{-1}$ (Table 5.1). Potassium nitrate (KNO_3) was used as the N source. To ensure adequate N supply, the soybean plants were fertilized with modified Hoagland solution and the control plants were supplied with modified N free Hoagland solution. The treatments were arranged in a completely randomized design (CRD) with six replicates in the growth chamber.

Table 5.1 Growth chamber N treatments

N rates	0 kg ha^{-1}	10 kg ha^{-1}	20 kg ha^{-1}	30 kg ha^{-1}
N fertilizer g $plant^{-1}$	0	0.020	0.040	0.061
mg $plant^{-1}$	0	20.24	40.48	60.72

5.3.1 Plant Materials and Inoculation

The growth chamber experiment started on January 26, 2011 in the Department of Plant and Animal Sciences at the Nova Scotia Agricultural College. Prior to the experiment, the growth chamber was cleaned using a bleaching solution to minimize the contamination. The 2 L pots were filled with 750-800 g of Pro-mix BX *(Premier Horticulture, Canada)* wetted with water. Slurry was prepared by mixing the inoculant with distilled water. Four hours prior to sowing, the seeds were mixed with the slurries. As the control treatment, the same volume of distilled water used for inoculated treatments was added to the seeds. The seed sowing began with the un-inoculated seeds and continued with the higher rates of inoculant. Also, from one treatment to the other treatment, hands were washed with 75% ethanol (C_2H_5OH). The experiment was carried out in the growth chamber under the conditions of day and night temperatures of 26/20 ^{o}C, relative humidity of 80% and day light intensity of 300-400 μmol m^{-2} s^{-1}. One week after seedling emergence, the plants were thinned by keeping one healthy plant per pot. The evapo-transpiration rate was measured in each pot by measuring the weight reduction 24 hours after watering.

5.3.2 N Treatment Application Method

The N treatment application started 15 DAP (once the roots had developed) to make sure that the soybean plant took up the entire applied N. The different rates of N were divided into three portions and 33% of the treatment was applied to plants every second day. This ensured efficient N uptake by the plant. Prior to application, the required quantities of KNO_3 were mixed with 25 ml of N free Hoagland solution and kept

for half an hour to dissolve the particles. For the control treatment, 25 ml of N free Hoagland solution was applied. The N free Hoagland solution (Hoagland and Arnon, 1950) consists of, 5 mM KH_2PO_4, 2 mM $MgSO_4$, 2.5 mM $CaSO_4$ and micronutrients of 46 μM H_3BO_3, 0.3 μM $CuSO_4$, 0.8 μM $ZnSO_4$, 9 μM $MnCl_2.4H_2O$, 0.1 μM $H_2MoO_4.H_2O$, and 89 μM Fe-EDTA (pH 5.5-5.8). Each plant was supplemented with 25 ml of Hoagland solution once every 2 days.

5.3.3 Plant Sampling and Measurements

The leaf photosynthesis rates and the chlorophyll contents were measured at 2-3 week intervals to observe the effects of applied treatments on photosynthesis and chlorophyll content. The photosynthesis rates of the fully expanded youngest mature leaf were measured by using the LCi portable photosynthesis system *(ADC BioScientific Ltd.)*. The measurements included transpiration (E), stomatal conductance (Gs), intercellular CO_2 concentration (Ci) and instantaneous carboxylation efficiency (A/Ci). Similarly, the chlorophyll content was measured in youngest mature leaf of each soybean plant by using a hand held chlorophyll content meter 200 *(CCM200, Opti-sciences, INC, NH, USA)*

Plant samples were obtained at mid pod filling stage (75 DAP) and at harvest maturity stage. Three plants (replicates) were uprooted at each sampling. As the peak N fixation of the soybean plant is at the mid pod filling stage, the sampling was carried out at this stage to observe the treatment effects on nodulation. The PFW, PDW, nodule numbers, nodule weights, root fresh weights (RFW), root dry weights (RDW) and the

pod numbers were measured at 75 DAP. The uprooted plants were divided into leaves, roots, and pods and they were oven dried at 60 $^{\circ}$C to a constant weight.

5.3.4 Extraction of Stem Ureide Compounds

The extraction of stem ureide N compound was carried out as described in the Chapter III section 3.2.5.2. The ureide-N concentrations were measured by the Young and Conway method (1942), and NO_3-N by the Cataldo method (Cataldo et al., 1974) as described in the Chapter III section 3.2.5.2.

5.3.5 Statistical Analysis

The experimental design was a two-factor factorial arranged in a completely randomized design with two factors. Before running the ANOVA, the normality and constant variance were checked by using Minitab 15 statistical software. The independence was assumed through randomization. The PROC MIXED procedure was used in SAS 9.2 statistical software for analysis. The statistical significance criteria was a Type III error rate of $P = 0.05$ with 95% confidence interval. LS means (Least Square Means) were used as the multiple mean comparison method when the effects were significant.

5.4 RESULTS

5.4.1 Effects of Rates of Inoculant and Starter N on Soybean Plant at Mid Pod Filling Stage under Controlled Environment Conditions

The results of the ANOVA Table 5.2 show the effects of rates of inoculant and the rates of N fertilizer on PFW, PDW, RFW, RDW and PN at 75 DAP. According to the table, PFW was significantly increased with N rates compared to the non-fertilized plants (Figure 5.1). However, increasing N supply from 40 mg of N plant^{-1} to 60 mg of N plant^{-1} did not increase the PFW. The interaction effect of rates of inoculant and the rates of N was significant on PDW and PN. However, RFW did not vary among the treatment while the RDW was significantly influenced by the rates of inoculant.

Table 5.2 ANOVA table for inoculant and N treatments on plant fresh weight, plant dry weight, root fresh weight and pod number at 75 DAP

Variation	Plant fresh weight (g)	Plant dry weight (g)	Root fresh weight (g)	Root dry weight (g)	Pod number
I	0.2643	0.0367*	0.4259	0.0371*	0.0001**
N	0.0395*	0.0057**	0.8578	0.8945	0.0103*
I× N	0.2416	0.0183*	0.3512	0.1398	0.0038**
I_0 vs $I_{1.5}$,I_3& $I_{4.5}$	0.1622	0.0398*	0.2207	0.0197*	0.0009**
$I_{1.5}$ vs I_3& $I_{4.5}$	0.1621	0.0297*	0.5985	0.2099	0.0002**
I_3 vs $I_{4.5}$	0.7962	0.7449	0.3244	0.1443	0.6511
N_0 vs N_{20},N_{40}& N_{60}	0.0106*	0.0009**	0.4533	0.7113	0.0060**
N_{20} vs N_{40}&N_{60}	0.1762	0.5334	0.7597	0.8610	0.0688
N_{40} vs N_{60}	0.6883	0.2292	0.7914	0.5223	0.1858

*and ** significant at 5 and 1% probability level, respectively.

Un-inoculated plants and the inoculant rate IR1.5 increased the PDW linearly with the N rate (Figure 5.2). The highest PDW was observed in treatment combinations of IR3-N20 and IR.5-N40. The PDW declined in inoculant rates of IR3 and IR4.5 beyond

the N rates of N20 and N40, respectively. Although RFW was not significantly affected by inoculation and N application, the RDW was significantly reduced with the rates of inoculant. The lowest RDW was observed in IR4.5 (Figure 5.3).
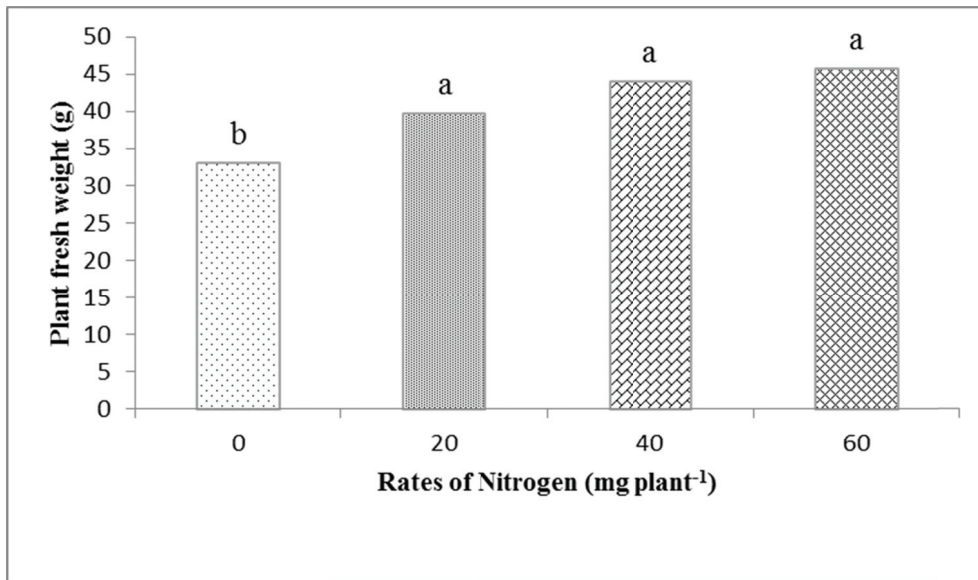
Figure 5.1 Effect of N supply on plant fresh weight at the mid pod filling stage.
Observations with the same letter are not significantly different.
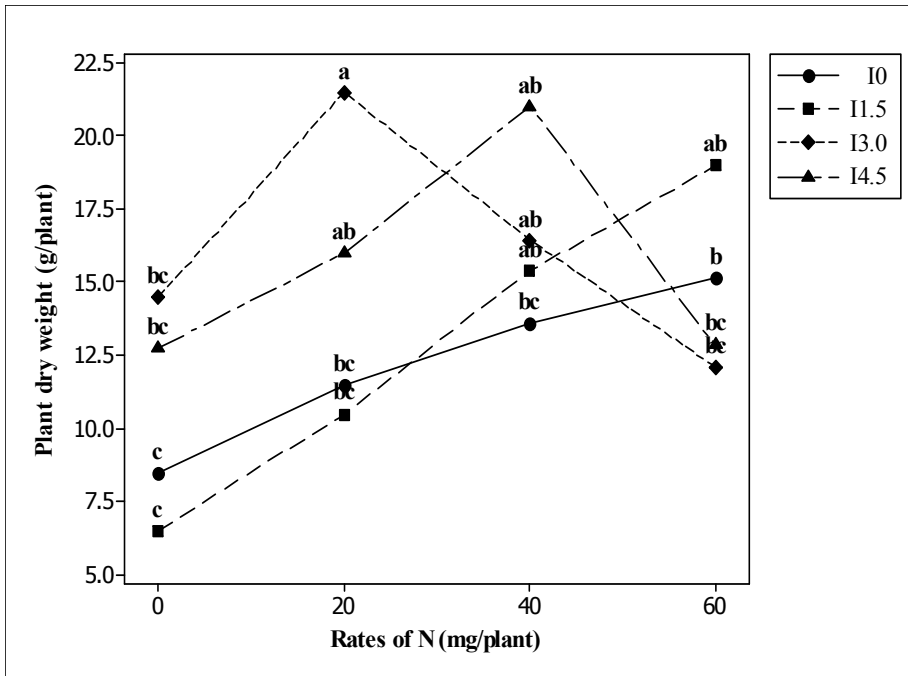
Figure 5.2 Plant fresh weights as affected by interaction effects of inoculation and N rates at the mid pod filling stage. Observations with the same letter are not significantly different.
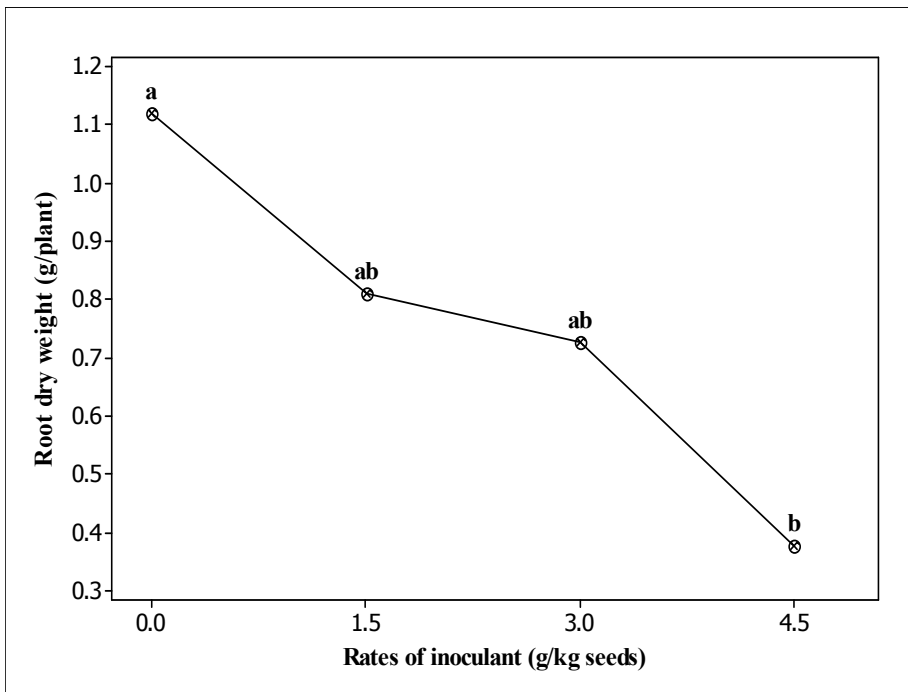
Figure 5.3 Root dry weights as affected by interaction effects of inoculation and N rates at the mid pod filling stage. Observations with the same letter are not significantly different.
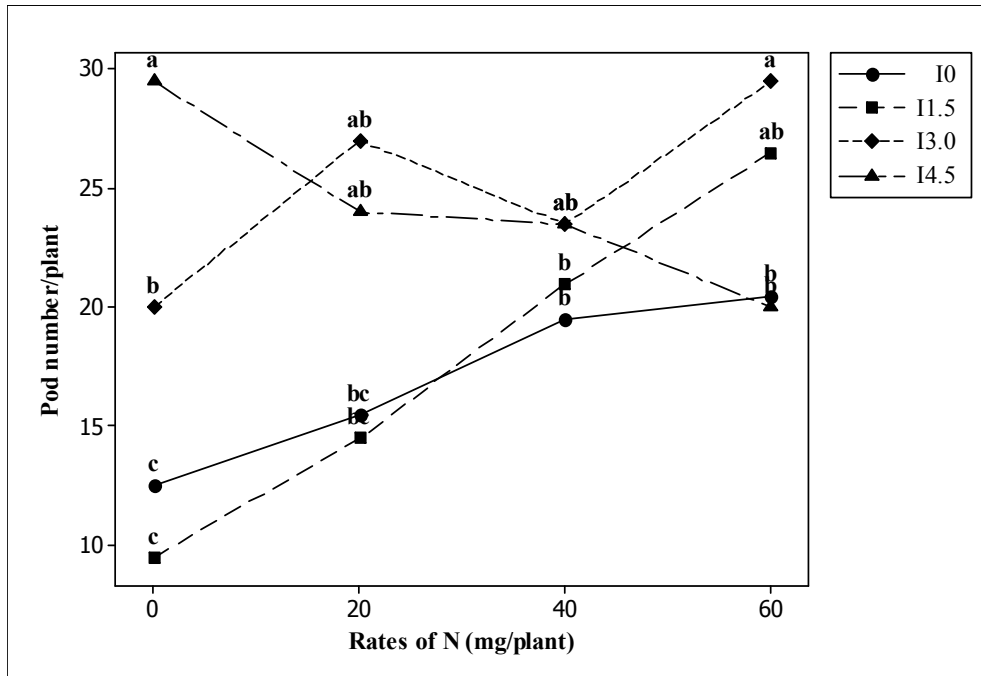
Figure 5.4 Pod number as affected by interaction effects of inoculation and N rates at the mid pod filling stage. Observations with the same letter are not significantly different.

The inoculant rate of IR4.5 showed significantly higher number of pods with N0, N20 and N40 (Figure 5.4). Significantly, a lesser number of pods was produced by the IR4.5 with N60. For IR3, a significantly higher number of pods was observed with N60, N40, and N20. However, IR3 without N supply produced significantly fewer pods compared to the IR3 treatment, combined with N. The pod numbers of IR1.5 and IR0 were linearly elevated with increasing N supply.

5.4.2 Effect of Rates of Inoculant and Starter N Application on Soybean Nodulation under Controlled Environment Conditions

ANOVA results in Table 5.3 indicate the effects of rates of inoculant on nodule numbers and nodule weights. There were no interaction effects of rates of inoculant and

N on nodule number and the nodule fresh weight. However, the rates of inoculant significantly affected the nodule number and fresh weights.

Table 5.3 ANOVA table for rates of inoculant on plant nodule number and nodule weight

Variation	Nodule number	Nodule fresh weight
I	<0.0001**	0.0125**
N	0.8954	0.4218
I× N	0.9318	0.6853
I_0 vs $I_{1.5}$,I_3& $I_{4.5}$	<0.0001**	0.0020**
$I_{1.5}$ vs I_3& $I_{4.5}$	0.1205	0.5571
I_3 vs $I_{4.5}$	0.6194	0.3210

*and ** significant at 5 and 1% probability level, respectively.

Although the inoculated treatments produced a significantly greater number of nodules compared to the control plants (Figure 5.5), nodule number of IR1.5, IR3, and IR4.5 treatments did not significantly vary.

The fresh nodule weight was inversely related to the nodule number and greater fresh nodule weight was observed for IR1.5 and IR3 than IR4.5 without any significant difference (Figure 5.6). At higher rates of inoculant, the plants tended to produce greater number of nodules that were less than 2 mm in diameter (Figure 5.7). However, the response of nodule diameters to the applied rates of N was inconsistent.
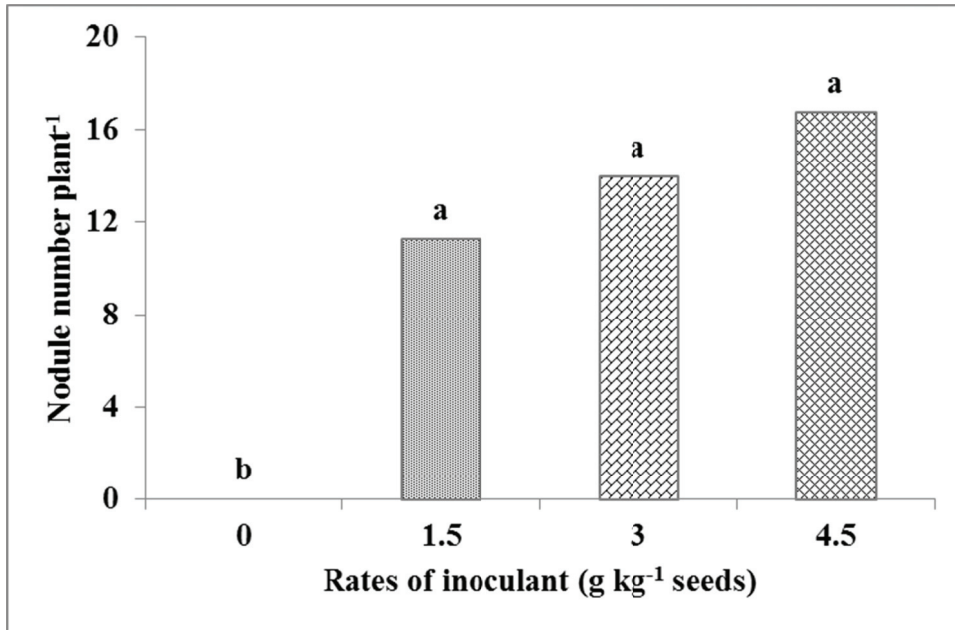
Figure 5.5 Effect of rates of inoculant on nodule number at the mid pod filling stage. Observations with the same letter are not significantly different.
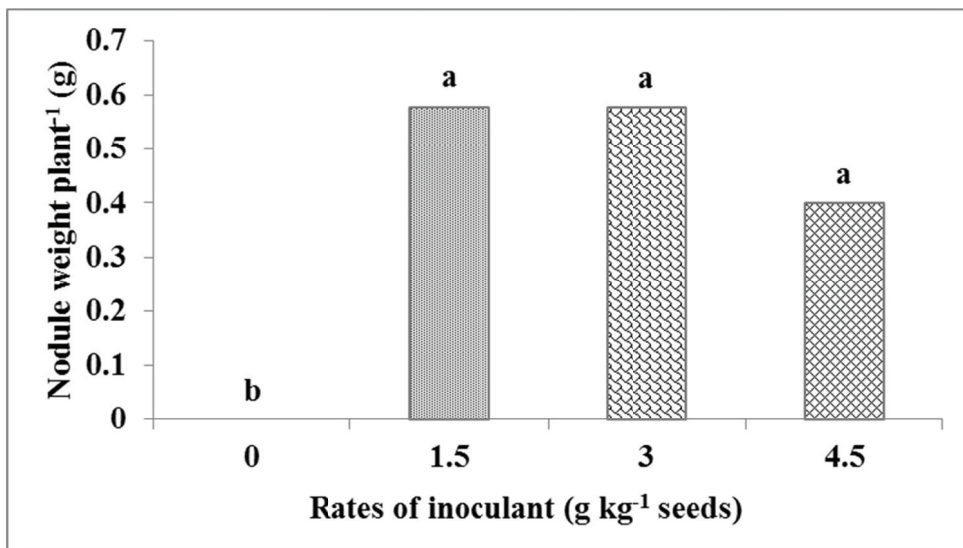
Figure 5.6 Effect of rates of inoculant on nodule fresh weight at the mid pod filling stage. Observations with the same letter are not significantly different.
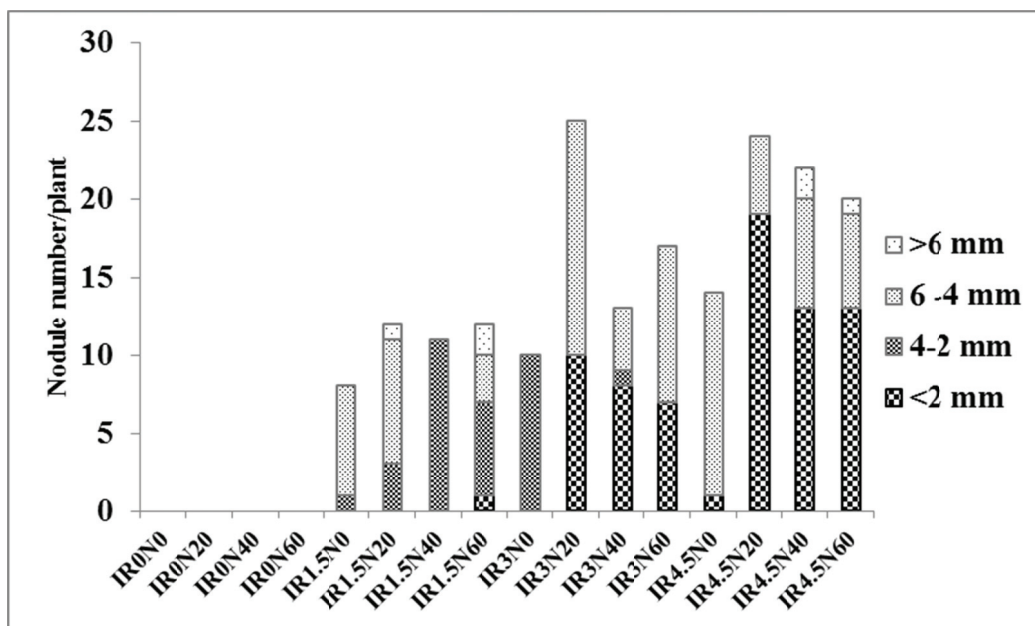
Figure 5.7 Nodule distribution based on the nodule diameter

5.4.3 Effect of Rates of Inoculant and Starter N Application on Soybean Seed Yield under Controlled Environment Conditions

The interaction effect of inoculation and starter N application on total plant weight, empty pod weight, and seed weight at harvest was significant (Table 5.4). With the exception of IR1.5 treatment, there was no significant interaction effect on the total plant dry weight. The plant weight of the treatment IR0-N40, IR1.5-N40, IR1.5-N60, IR3-N20, and IR3-N60 was significantly greater. Similarly, the empty pod weight was significantly higher in IR1.5 with N rate of N60. For the IR3 treatment, the highest empty pod weight was observed at N0 level, while IR0 and IR4.5 did not show much variation with respect to the fertilizer N application.

The seed weight increased with N fertilizer application in the IR4.5 treatment. However, the seed weight did not linearly increase with N levels. The IR4.5 with N40 produced greater seed weight compared to the other treatment combinations. The seed weight of the IR3 was not significantly different at N0, N20, and N60 levels and there

was a significant reduction in the seed weight of IR3 treatment at N40. The seed weight

of lower inoculant rate (IR1.5) plants increased with N40 and N60. The N application did

not significantly enhance the seed weight of the un-inoculated plants.

Table 5.4 Soybean plant weight, and seed weight at harvest

Means	Plant weight (g)	Empty pod weight (g)	seed weight (g)
IR0N0	15.67 bc	1.72 c	2.45b
IR0N20	16.00 b	2.11 c	2.64b
IR0N40	21.67 ab	3.02 bc	3.46b
IR0N60	13.67 bc	2.28 c	3.24b
IR1.5N0	7.77c	1.1 c	3.01b
IR1.5N20	6.67 c	2.07 c	3.016b
IR1.5N40	24.33 a	3.54 bc	5.22ab
IR1.5N60	20.67 ab	7.40 a	6.40ab
IR3N0	14.00 bc	5.50 ab	7.57ab
IR3N20	19.33 ab	4.85 b	7.55ab
IR3N40	7.67 c	2.58 bc	3.03b
IR3N60	22.67 ab	4.62 bc	7.33ab
IR4.5N0	14.00 bc	2.45 bc	4.87b
IR4.5N20	13.67 bc	4.65 bc	7.34ab
IR4.5N40	16.67 b	4.21 bc	7.96a
IR4.5N60	14.67 b	3.96 bc	7.70ab
Variation			
I	0.6451	0.0134*	<0.0001**
N	0.0145*	0.0363*	0.1417
I× N	<0.0001**	0.0038**	0.0258*

Observations with the same letter are not significantly different.

5.4.4 Effect of Rates of Inoculant and Starter N Application on Soybean Leaf Chlorophyll and Photosynthesis Rate under Controlled Environment Conditions

At the beginning of fertilizer N application, the leaf chlorophyll content at 30

DAP (Figure 5.8) was significantly affected by the rates of inoculant and the rates of N

fertilizer (Table 5.5). However, at 45 DAP, the leaf chlorophyll content was marginally

significant with the rates of inoculant while at late pod filling stage (90 DAP), the leaf

chlorophyll content was significantly increased in the inoculated plants (Figure 5.9). The

un-inoculated plants started to shed their leaves earlier than the inoculated plants.

Table 5.5 ANOVA table for rates of inoculant and starter N on leaf chlorophyll content

Variation	Chlorophyll content 30 DAP	Chlorophyll content 45 DAP	Chlorophyll content 90 DAP
I	<0.0001**	0.0541*	0.0029**
N	0.0414*	0.1170	0.3014
I× N	0.0047**	0.7043	0.3774

*and ** significant at 5 and 1% probability level, respectively.

Even though the interaction effect was significant, the leaf chlorophyll content of

IR3 and IR4.5 treatments were significantly greater without any N fertilizer at 30 DAP.

The leaf chlorophyll content of IR1.5 was significantly higher with N40 than N0 level at

30 DAP. Greater levels of chlorophyll content were observed in IR1.5, IR3 and IR4.5

without N application (Figure 5.9) at 90 DAP.
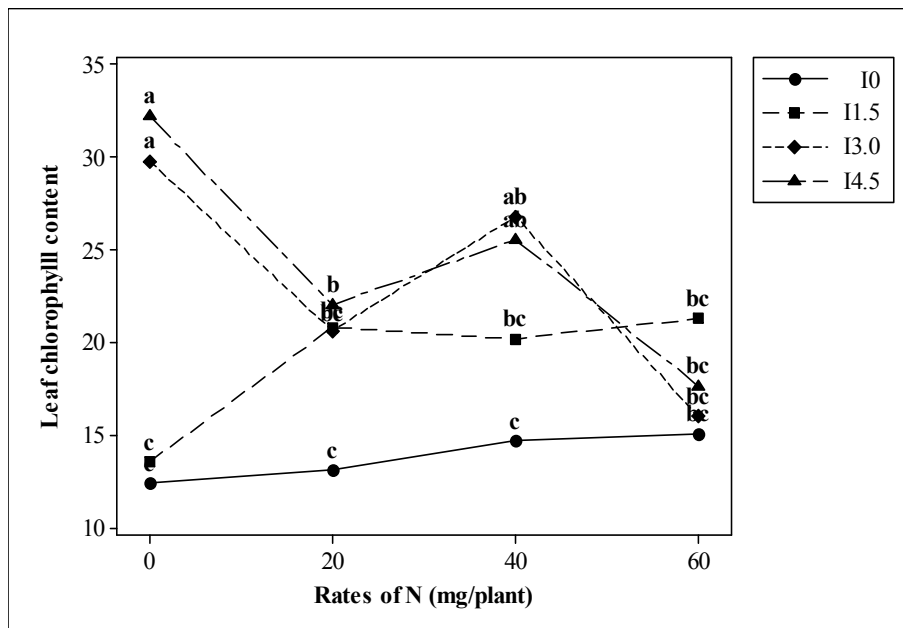
Figure 5.8 Leaf chlorophyll contents as affected by interaction effects of inoculation and N rates at 30 DAP. Observations with the same letter are not significantly different.
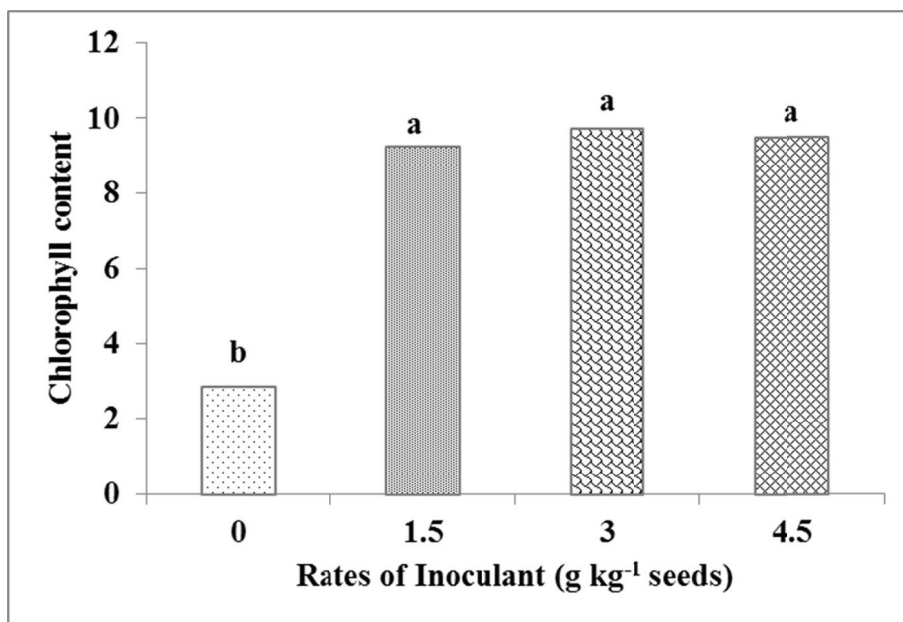
Figure 5.9 Leaf chlorophyll contents as affected by inoculation at 90 DAP. Observations with the same letter are not significantly different.

The results in the ANOVA Table 5.6 indicate that the interaction effect of the rates of inoculant and the rates of N fertilizer was significant on most of the photosynthesis variables at 30 DAP; photosynthesis rates (A), intercellular CO_2 (Ci) and the transpiration rate (E). The photosynthesis rates were significantly increased in the un-inoculated plants with applied rates of N fertilizer up to N40 (Figure 5.10). N application did not significantly increase the photosynthesis rates of inoculated plants.

Table 5.6 ANOVA table for rates of inoculant and starter N on photosynthesis measurement at the vegetative stage (30 DAP)

Variation	A	Ci	E
I	<0.0001**	0.0266*	0.0463*
N	0.0004**	0.1527	0.6498
I× N	<0.0001**	0.0248*	0.0004**

*and ** significant at 5 and 1% probability level, respectively.
Photosynthesis rates (A), intercellular CO_2 (Ci) and the transpiration rate (E)

Similarly, intracellular CO_2 (Ci) was greater in the un-inoculated plants compared to the inoculated plants with respect to the N supply (Figure 5.11). The significant Ci concentration was observed in IR0 with the higher N level (N60). The leaf transpiration rates (E) were high in both IR4.5 and IR0 with N0 treatment (Figure 5.12). However, E increased linearly in IR1.5 and IR0 treatments with N supply. The E value of the IR0-N60 treatment combination was significantly high. The effects of photosynthesis variables Ci did not alter with the treatments (Table 5.7) at mid pod filling stage (75 DAP). Rates of inoculant significantly increased (Figure 5.13) A and E (Figure 5.14) in inoculated plants compared to the un-inoculated plants. Also, there was a marginally significant effect of inoculant on photosynthesis rate.

Table 5.7 ANOVA table for rates of inoculant and N on photosynthesis measurement at the mid pod filling stage (75 DAP)

Variation	A	Ci	gs	E
I	0.0489*	0.4147	0.2476	0.0199*
N	0.4777	0.9924	0.3899	0.9633
I× N	0.9246	0.3596	0.8532	0.2228

*and ** significant at 5 and 1% probability level, respectively.
Photosynthesis rates (A), intercellular CO_2 (Ci), transpiration rate (E) and stomatal conductance (Gs).
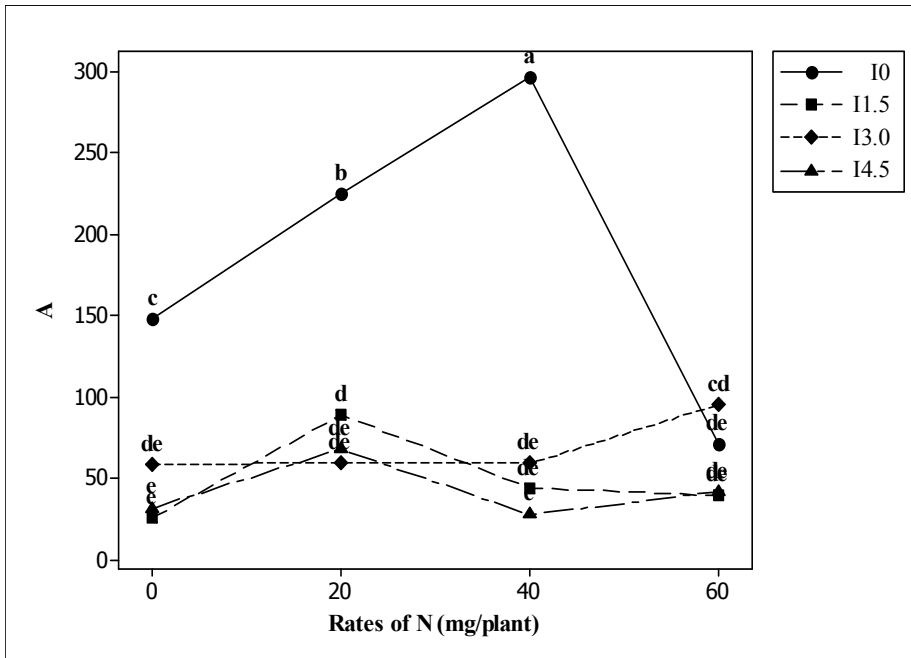
Figure 5.10 Leaf photosynthesis rates (A) (µmol m^{-2} s^{-1}) as affected by interaction effects of inoculation and N rates at 30 DAP. Observations with the same letter are not significantly different.
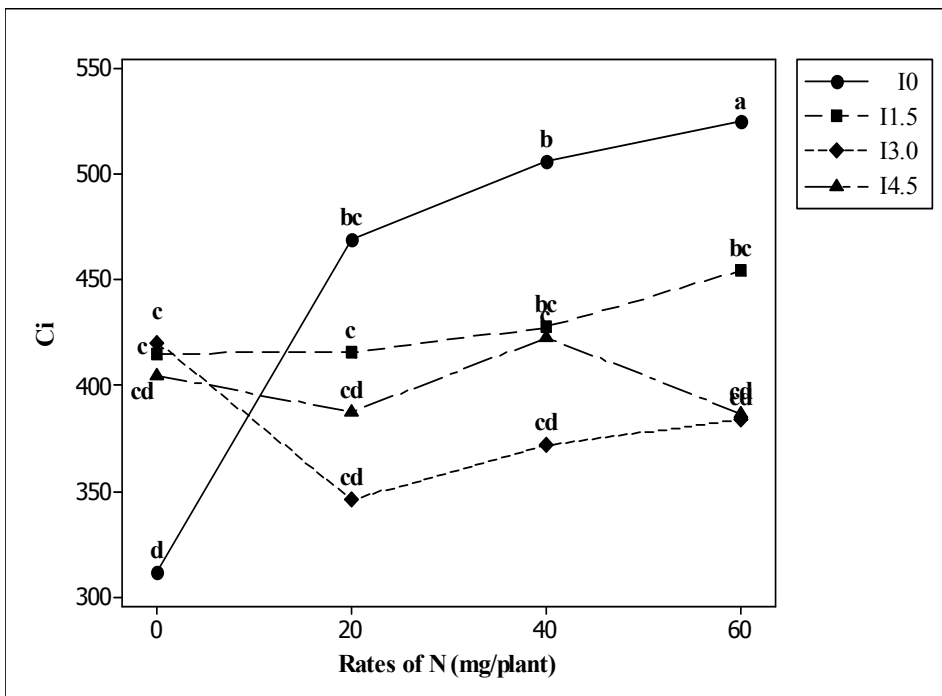
Figure 5.11 Leaf intracellular CO2 (Ci) (vpm) as affected by interaction effects of inoculation and N rates at 30 DAP. Observations with the same letter are not significantly different.
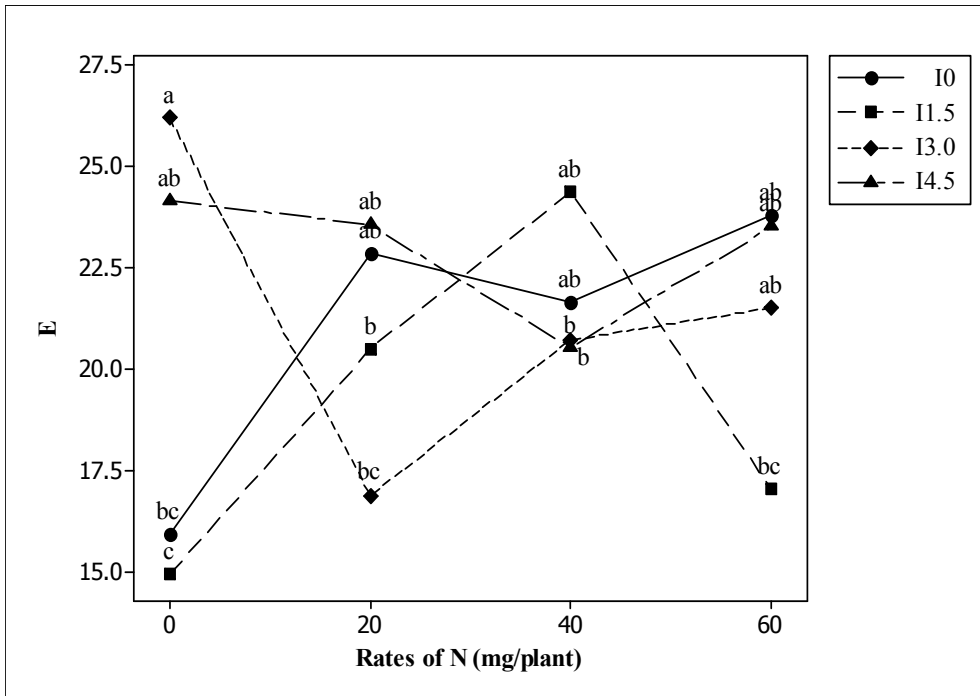
Figure 5.12 Leaf transpiration rates (E) (μmol m^{-2} s^{-1}) as affected by interaction effects of inoculation and N rates at 30 DAP. Observations with the same letter are not significantly different.
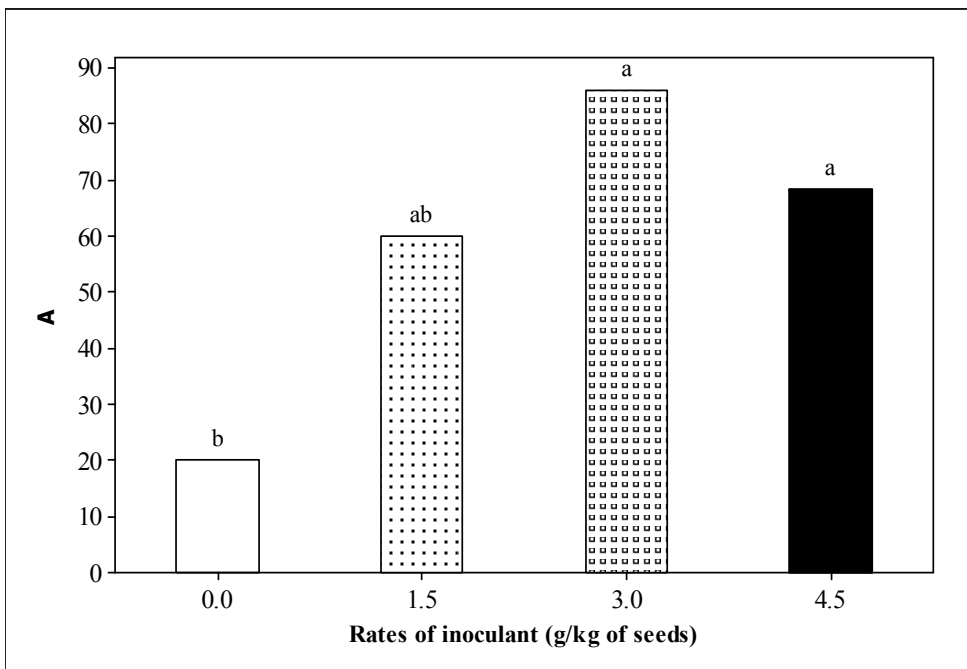
Figure 5.13 Effect of rates of inoculant on leaf photosynthesis rates (A) (μmol m^{-2} s^{-1}) at 75 DAP. Observations with the same letter are not significantly different.
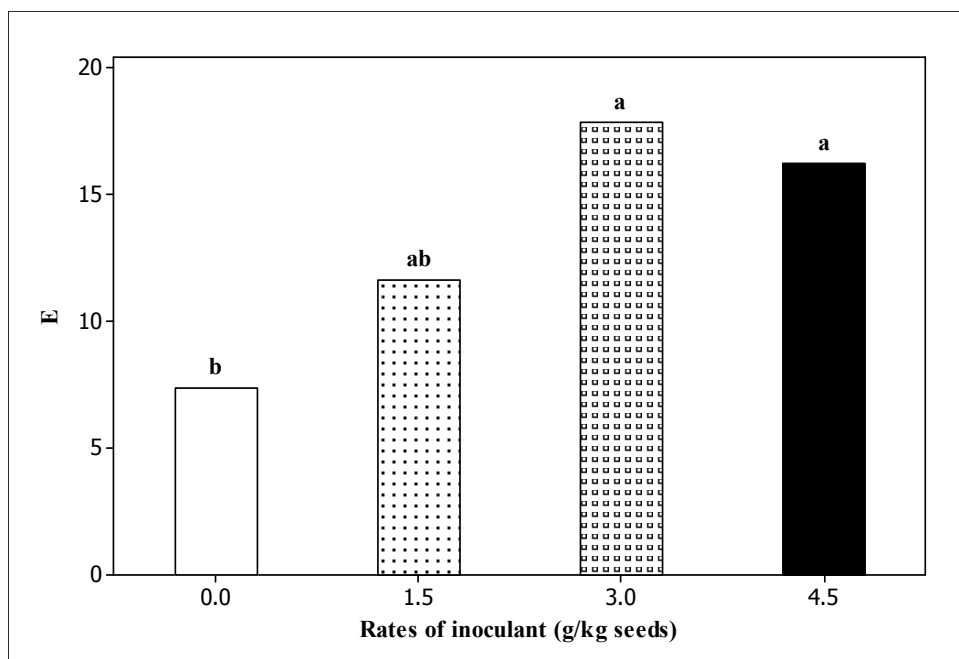
Figure 5.14 Effect of rates of inoculant on leaf transpiration rates (E) (μmol m^{-2} s^{-1}) at 75 DAP. Observations with the same letter are not significantly different.

5.4.5 Effect of Rates of Inoculant and the Starter N Application on Soybean N Fixation under Controlled Environment Conditions

The stem NO_3-N and the ureide-N were determined in order to evaluate symbiotic N fixation responses. The stem NO_3-N was not affected by the N supply (Table 5.8). However, the ureide-N content and the RU% were significantly influenced by the interaction effect of inoculation and the N supply. The amino-N levels were significantly affected by inoculation and N supply.

The stem ureide-N content was significantly lower in the un-inoculated plants compared to all inoculant levels (Figure 5.15). The treatment combinations IR1.5-N0 and IR3-N60 had the higher ureide-N concentrations. Application of N fertilizer did not affect stem ureide-N concentrations of IR4.5.

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Table 5.8 ANOVA table for rates of inoculants and N on photosynthesis measurement at mid pod filling stage

Variation	NO_3-N	Ureide-N	Amino-N	RU%
I	0.1060	<.0001**	0.0009**	<.0001**
N	0.3244	<.0001**	0.0082**	0.0077**
I× N	0.0828	<.0001**	0.1406	0.0074**
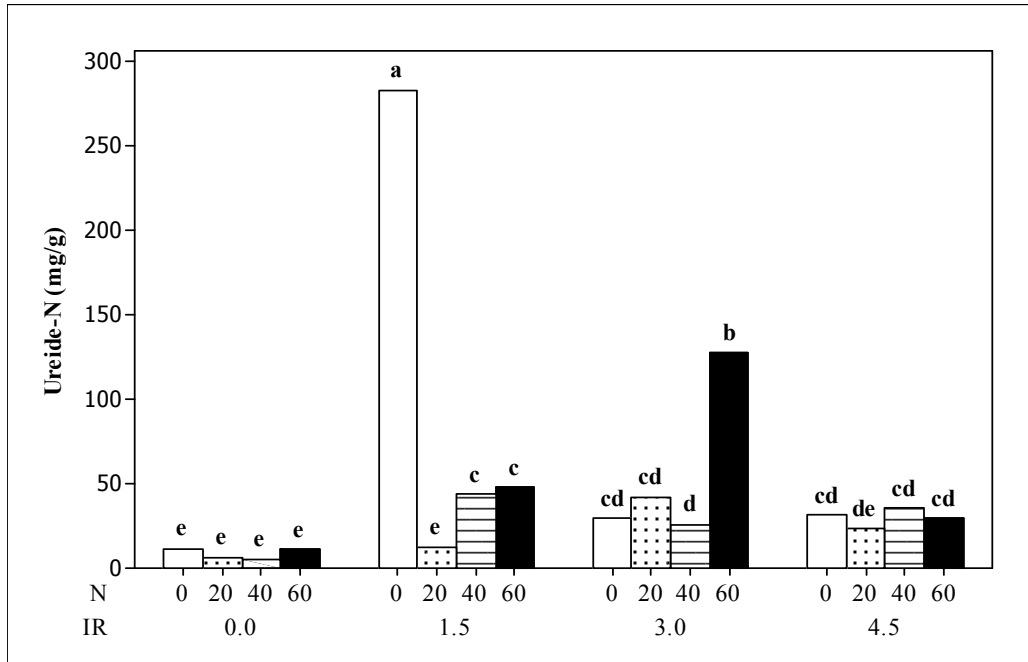
Figure 5.15 Interaction effects of rates of inoculant and N supply on stem ureide concentrations at 75 DAP. Observations with the same letter are not significantly different.

The RU% of IR1.5 was highest with N0 (Figure 5.16) and there was a significant reduction in RU% from N20 to N60. In the IR3 and IR4.5 treatments, there was no difference in RU% with N fertilizer application. The RU% of the un-inoculated plants with N60 was higher than those of N0 to N40.
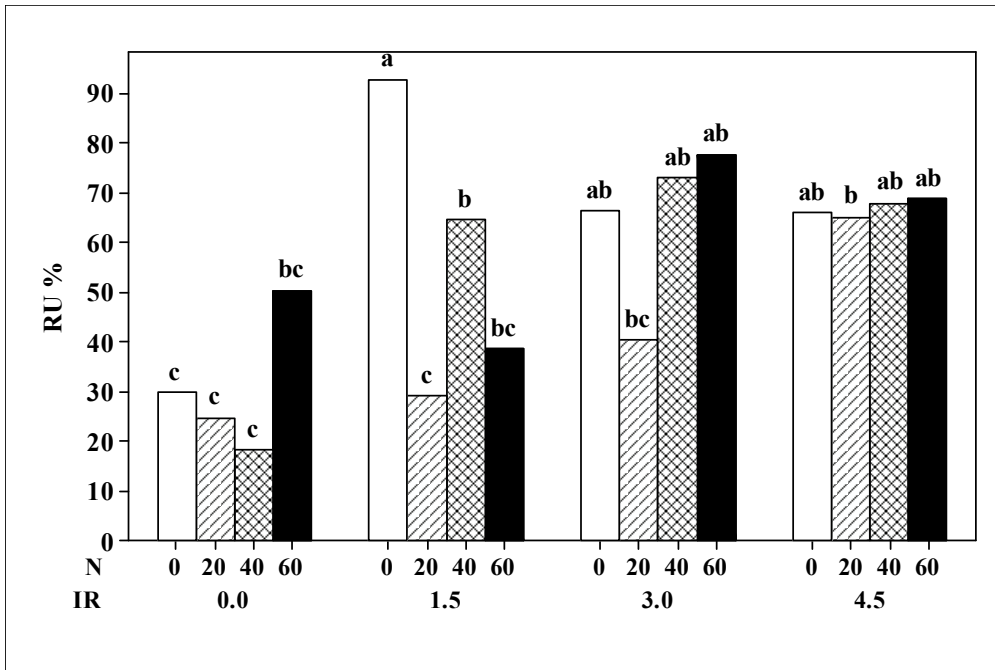
Figure 5.16 Interaction effect of rates of inoculant and N supply on RU% at 75 DAP. Observations with the same letter are not significantly different.

5.5 DISCUSSION

5.5.1 Soybean Nodulation

Soybean nodulation was significantly elevated in inoculated plants, compared to the control plants. There was an increasing trend in nodule number with increasing *Bradyrhizobium* cell numbers per seed in this study. The same trend was also reported in previous researches (Buwaneswari et al., 1988; Brockwell et al., 1989; Papakosta, 1992). In the present experiment, nodule numbers were proportionate to applied rates of inoculant, but the weight of nodules was inversely related. Buwaneswari et al. (1988) also reported that nodule formation increased with inoculant dosage. Higher rates of inoculant increased the number of bacterial cells in the rhizosphere and their multiplication, which increased nodule formation, result in larger numbers of nodules per plant. The present study has found that greater numbers of nodules require larger amounts of energy and space to develop. These two factors may be the reason that a large number of smaller nodules was observed in the current study. Papakosta (1989) stated that an increase in nodule number per soybean plant reduced the nodule size. Accordingly, a similar pattern was observed in the current growth chamber study.

In agreement with Diep et al. (2002), the present study also observed no effect of N fertilizer application on soybean nodulation. Under field conditions, they reported that the application of a small amount (25 kg ha^{-1}) of fertilizer N did not suppress soybean nodulation. The rates of N fertilizer used in the growth chamber study were 0, 20, 40 and 60 mg plant^{-1}, which correspond to 0, 10, 20 and 30 kg ha^{-1} , respectively, in the field study. This is the only N source used in the growth chamber except 70-130 mg l^{-1} N present in the Pro-mix (www.growercentral.com). Unlike the soil, there was no rapid

mineralization in Pro-mix medium and nutrients became more and more limited with time. These observations implied that the application of N fertilizer could not suppress soybean nodulation with low amounts of available growing medium N.

5.5.2 Soybean Plant Growth and Yield

In the current study, the soybean plant growth and seed yield were significantly affected by the interaction between the rates of inoculant and the rates of starter N fertilizer. Taylor et al (2005) found that the application of fertilizer N promoted soybean plant growth and seed yield up to 60-70 kg ha^{-1}. In the growth chamber study, the highest seed weights for IR4.5 and IR1.5 were observed in N applied plants while IR3-N0 produced the same amount of yield as the above treatments. There are many previous researches, which concluded that the application of N fertilizer can boost soybean plant yield (Taylor et al., 2005; Morshed et al., 2008).

Restricted root growth was observed in the inoculated plants and IR4.5 had the lowest RDW. Some studies have reported that super nodulating soybean lines have restricted root growth that limited nutrient absorption (Ohayama et al., 1993; Matsunami et al., 2004). Also, restricted root growth in the plants relying on N fixation was (Pate et al., 1979) suggested due to higher demand of photosynthate (Russell and Johnson, 1975). This could be the reason for the suppression of root growth at higher inoculant rates.

5.5.3 Effects on Chlorophyll Content and Photosynthesis

Leaf chlorophyll content was increased following inoculation and fertilizer N application at the early vegetative growth stage (30 DAP). The leaf chlorophyll content of the un-inoculated plants and the lowest rate of inoculant (IR1.5) increased with the N fertilizer application at 30 DAP. At the late pod filling stage (90 DAP), inoculation alone had a significant effect on the leaf chlorophyll content. N fertilizer application and soybean inoculation increased the leaf chlorophyll content and plant biomass. Kaschuk et al (2009) reported that the leaf chlorophyll content of nodulated plants remained at high levels until the pod filling stage, while the chlorophyll content of N fertilized plants started to diminish at the flowering stage. The same phenomenon was observed in the present study. N is a major component of the leaf chlorophyll. However, inoculated plants alone provided sufficient levels of N to enhance the chlorophyll content at 90 DAP.

The photosynthetic rates did not show consistent response to rates of N fertilizer in all inoculated plants at the vegetative stage. However, at 30 DAP the photosynthetic rate rapidly increased in the un-inoculated plants up to N 40 level and these findings agreed with those of Zhou et al., (2006). At the pod filling stage, the leaf photosynthetic rates and the chlorophyll content was significantly higher in the inoculated plants compared to the un-inoculated plants. Kaschuk et al (2009) demonstrated that soybean plants with effective N fixing nodules have greater levels of photosynthesis compared to N fertilized plants. Studies have revealed that N fixation enhances the photosynthetic capability of legume plants (De Veau et al., 1990: Zhou et al., 2006). The carbon cost of N fixation is higher than the cost of NO_3^- uptake. In symbiosis, *Rhizobia* utilize 4-16% of

recently fixed photosynthesis C to maintain their activity. Due to the C sink strength of symbioses, photosynthesis may be increased (Kaschuk et al., 2009). In the current experiment, the photosynthetic rates of the un-inoculated plants were less than the inoculated plants at 75 DAP and this emphasizes that nodulated plants have a higher demand for photosynthesis. The un-inoculated plants started to demonstrate senescence earlier than the inoculated plants and this trend was also observed by Kaschuk et al (2009). The delay of leaf senescence in nodulated plants could be associated with the higher photosynthesis rates.

5.5.4 Soybean Symbiotic N Fixation

The RU%, calculated was based on the stem ureide-N and NO_3-N concentrations. The RU% values obtained in this study were in the range of Herridge and People, (1990). A two-way interaction was observed for the stem ureide-N concentration and RU%. There was no consistent effect on stem ureide-N levels with the rates of inoculant and the rates of N fertilizer. Since the study was conducted in a growth chamber, there was a minimum number of factors which influenced plant N uptake. The current study reveals that application of N fertilizer to a growing medium with inadequate amounts of N cannot suppress symbiotic N fixation as the RU% did not vary in the inoculant rates 3 and 4.5 g kg^{-1} seed with respect to N application. However, fertilizer N reduced the efficiency of symbiotic N fixation with lower rates of inoculant. According to Salvagiotti (2009), the results in the current study could be due to the early surface applications of fertilizer N. This can suppress the symbiotic N fixation during the entire crop cycle.

5.6 CONCLUSION

Early application of starter N fertilizer increased the plant biomass with the inoculant rate of 1.5 g kg^{-1} seeds or without inoculant. Significantly greater seed yield was obtained with an inoculant rate of 1.5 and 4.5 g kg^{-1} seed with N applied plants while inoculant rate 3 g kg^{-1} seed rate without N fertilizer also produced the similar amount of seed yield under controlled environmental conditions. Application of low levels of starter fertilizer N to a growing medium with inadequate amounts of available N did not suppress the soybean plant's symbiotic N fixation. The inoculated plants alone increased the leaf chlorophyll content and the photosynthetic rates in soybean at the pod filling stage.

CHAPTER 6   CONCLUSIONS

The research was conducted to achieve the main goal of improving soybean symbiotic N fixation and grain yield in the dykeland soils. In order to fulfill the above research objective, several secondary objectives were set. At the very beginning, the focused was on isolating *Rhizobia* from the dykeland soils. Then, a two-site study was conducted to evaluate the soybean symbiotic N fixation and yield responses in relation to the rates of inoculant and the starter N fertilizer in dykelands. In addition to this field study, a growth chamber study was set up to observe the effects of rates of inoculant and starter N on soybean N fixation, leaf chlorophyll, photosynthesis rate and grain yield.

6.1. ISOLATION OF *RHIZOBIUM* FROM DYKELAND SOILS

Depending on soil conditions, there was a great variability in dykeland *Rhizobial* population. The isolate W0 had enormous capability for soybean nodulation in the authentication study. Variation among isolates arose as a result of soil conditions (e.g. soil salinity) and the soil management practices (e.g. manure application).

Accurate strain identification is necessary to confirm the identity of isolates. Further studies on symbiotic N fixation are necessary in order to recommend the isolates for field use. For this, a comparison of N fixation between isolates and commercial inoculant is necessary.

6.2 N Fixation and Grain Yield Responses of Soybean in Relation to Rates of Inoculant and Starter Fertilizer N Application in Dykelands

It was concluded that application of commercial inoculant promotes soybean plant growth and seed yield under favourable dykeland soil conditions. The inoculated plants produced significantly greater grain yield compared to the un-inoculated plants. Soil salinity is one of the deleterious factors affecting soybean symbiotic N fixation on dykelands. Since these fields are susceptible to changes in soil conditions, dual inoculation of soybean with two *Rhizobial* strains can be more effective in terms of promoting symbiotic N fixation. Starter fertilizer N rates of 10, 20, and 30 kg ha^{-1} did not affect either soybean N fixation or grain yield significantly. However, at 30 kg N ha^{-1}, there was a substantial reduction in the nodule number in all inoculant treatments under acidic soil conditions. Application of starter N fertilizer at low levels (10, 20, and 30 kg ha^{-1}) may not be necessary for soybean cultivation.

The soybean plant N demand is greatest at the pod filling stage as the developing seeds act as a strong sinks. At this time, the vegetative plant parts also translocate N to developing seeds. N fertilizer application during the reproductive stage perhaps could have a positive effects on soybean yield. For instance, Gan et al (2003) reported that the most suitable timing for fertilizer N top dressing is during reproduction at the flowering stage. In future, it is recommended that the effects of N fertilizer top dressing at the reproductive stage in the dykelands be investigate. It has been shown that the amount of fertilizer N required to achieve better response is greater for surface application than deep placement of slow release N. Application of slow release fertilizer below the root nodulating zone before planting did not reduce the BNF (Salvagiotti, 2009; Tewari et al.,

2004). Therefore, evaluation of surface application versus deep placement of slow release N fertilizer with different rates will be useful in future studies. Conducting this kind of study in a wide range of dykeland soil and under various management conditions would be beneficial for developing N fertilizer strategy and recommendations.

6.3 N FIXATION AND GRAIN YIELD RESPONSES OF SOYBEAN IN RELATION TO RATES OF INOCULANT AND STARTER FERTILIZER N APPLICATION UNDER CONTROLLED ENVIRONMENT CONDITIONS.

Under controlled environmental conditions, fertilizer N promotes plant growth. However, 3 g kg^{-1} seed without N fertilizer also produced the same amount of grain yield as the inoculant rate 1.5 and 4.5 g kg^{-1} seed with N fertilizer. According to the stem RU%, starter N application to an agriculture system with low available N could not impart an adverse effect on soybean N fixation was indicated by stem.

Instead of using Pro-mix, use of dykeland soils as the growing medium could be beneficial to correlate the observation with dykeland soils. It also may helpful to recognize the soil chemical and biological factors that affect N fixation and grain yield in addition to soil conditions.

The overall conclusion of the research is that the inoculated plant produced greater grain yield compared to the un-inoculated plants. The inoculation of soybean with 150% of standard rate showed an increasing trend of grain yield in acidic dykeland soil conditions. However, the soil salinity appears to suppress N fixation under dykeland conditions in response to the commercial *Bradyrhizobium* inoculant. Starter fertilizer N was not effective in increasing soybean grain yield in dykelands.

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APPENDIX A: SOIL CLASSIFICATION FOR ACADIA SOILS (ACS)

Soil material : Fine loamy marine
Slope : 0.5% to 3%
Drainage : Imperfect to poor
Stoniness : Non stony
Rockiness : Non rocky

<u>**A horizon**</u>
Thickness : 10-51 cm
Particle size
Sand% : 4-19
Dominant sand size : Very fine
Silt% : 51-60
Clay% : 26-36
Hydraulic Conductivity (cm/h) : 0.1-10.8
Bulk density (g/cm^3) : 1.2-1.5
Organic carbon % : 0.6-2.2
pH (water) : 5.3-6.4
Consistence : friable to firm

<u>**C horizon**</u>
Particle size
Sand% : 4-27
Dominant sand size : Very fine
Silt% : 45-69
Clay% : 13-50
Hydraulic Conductivity (cm/h) : 0-29.3
Bulk density (g/cm^3) : 0.8-1.5
Organic carbon % : 0.7-2.8
pH (water) : 4.4-6.2
Consistence : friable to firm

Source: Holmstrom, D. A. 1989. Soils of the Annapolis Valley area of Nova Scotia.
Department of Agriculture and Marketing. Agriculture Canada.