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Evaluation The Nitrogen Needs And Efficiency Of Rizhobia Strains To Provide Nitrogen To Chipilin (Crotalaria Longirostrata Hook. And Arn.)

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EVALUATION OF THE NITROGEN NEEDS

AND EFFICIENCY OF RHIZOBIA STRAINS TO PROVIDE NITROGEN

TO CHIPILIN *(Crotalaria Longirostrata HOOK. AND ARN.)*

A Thesis Presented

by

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DEDICATION

To my loving mother.

ABSTRACT

EVALUATION THE NITROGEN NEEDS
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TO CHIPILIN *(Crotalaria Longirostrata HOOK. AND ARN.)*

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Chipilin (*Crotalaria Longirostrata*) is a leguminous plant native to Central America and Southern Mexico and used in the preparation of traditional dishes in this region. Starting in 2009, farmers in Massachusetts have been growing chipilin with a weekly production of 800 kg·ha^{-1}. However, as much as 300 kg·ha^{-1} of nitrogen has been necessary to apply to the soil in order to obtain a marketable leaf quality. With the goal to determine the nitrogen requirements of chipilín and to quantify the capacity of selected stains to infect and provide nitrogen for this crop, two-field experiments were conducted at the UMass Research farm at Deerfield, Massachusetts, in an occum fine sandy loam soil (coarse-loamy, mixed, mesic Fluventic Dystrudept) soil as a randomized complete bock design with five replications. For the field trial in 2011, nitrogen rates were (kg·ha^{-1}): 40, 80, 120, 160, 200 and 240 and 0, 40, 80, 120, 160, 200, 240 and 280 in 2012 in combination with four Rhizobia strains: *Bradyrhizobium sp. (Vigna), Rhizobium leguminosarum biovar, Bradyrhizobium* USDA 3384 and no *Rhizobia* were the treatments. Based on the results obtained, nitrogen fertilizer application of 80 kg·ha^{-1} was economically sufficient for chipilin to reach optimum yield. However higher nitrogen rates are needed to obtain marketable leaf color

and quality. Additionally a greenhouse experiment set up as a factorial experiment with five replications was conducted with seven nitrogen concentrations (mg N·L^{-1})$^-$; 0, 26.25, 52.5, 105, 157.5, 210 and 262.5 mg·L^1 and the three *Rhizobia* strain for the previous experiment plus *Bradyrhizobium* USDA 2370 as treatments. Results suggest from *Bradyrhizobium* USDA 3384 is not an efficient strain for chipilin, and *Rhizobium leguminosarum biovar* potentially may provide the most nitrogen of the strains evaluated. In the greenhouse trial, nodules number per plant decreased with the increase in nitrogen applications, but this was not the case in the field trial in 2012. Nodules were found on the root of chipilin plants in the control. This is suspected to be due to one of the following possibilities: Rhizobia inoculum presence in the seed, Rhizobia in the soil (in the field trial) or contamination during the setup of the experiment.

KEY WORDS: *Crotalaria longirostrata*, chipilin, rhizobia strain, nitrogen.

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CHAPTER 1

INTRODUCTION

Chipilin (*Crotalaria Longirostrata* Hook & Arn) is a legume originally from
Central America and southern Mexico, widely distributed in elevations of 1000 to 2200
meters above sea level (Chízmar, 2009). It is a leafy vegetable considered as the
Crotalaria species most edible of this genus (FAO, 2012). Raw chipilin leaves are
considered as purgative (Morton, 1994), but when cooked the young leaves and shoots
are widely used as leafy green in several traditional dishes of this region.

Chipilin has been considered as one of the 16 most important species of edible
leaves in the world (Arias*, et al.,* 2003) due its high content in calcium, iron, thiamine,
riboflavin, niacin and ascorbic acid (Morton, 1994) and as a valuable source of protein
(Arias*, et al., l* 2003). This species is also important as an efficient method for the control
of *Meloidogyne sp* populations in agricultural soils (Carraza, 2004).

Chipilin is an annual slender shrub that will sometimes persist for more than a
year. It grows upright to 0.6-1.8 meters high, with slender, vertical or arching branches,
nearly cylindrical, and slightly hairy. The stem often turns dark-red as it matures. The
closely-set, alternate leaves, on 18 mm petioles, have three short-stemmed, oblong-ovate
leaflets, 1.25-4 cm long, that are pale and silky on the underside (Figure 1). The pear-
shaped flowers borne on terminal racemes of 15 to 20 flowers that are yellow tinged with
brown spots that reach 2.5 cm long. The multi-ribbed pods of chipilin are narrow at the
base, rounded, with a sharp tip at the apex. At maturity, the pods are finely downy,
inflated and brown, and contain 4-6 seeds that are light-to dark-brown and over 3 mm

long. The pod split when fully ripe and the seeds are flung several feet away from the plant (Morton, 1994). It is propagated through seed during the rainy season in its the native countries (Chízmar, 2009). Seeds are considered toxic due the content of a carcinogenic alkaloid, presents in 20 percent of the species in this genus (Morton, 1994).

Figure 1.1 Symptoms of nitrogen deficiency in chipilín growing at the UMass-Research farm, South Deerfield,

As a result of the large population of Central Americans and Mexicans in the Northeastern of United States (Mangan, et. al. 2008), researchers at the University of Massachusetts have been evaluated chipilin since 2007 for production by farmers. As of 2012, several farmers are growing chipilin with an estimated weekly production of 800 kg·ha^{-1} and a wholesale price of $8.83 kg^{-1}.

In the Northeastern United States, chipilín is grown as an annual plant during the frost-free periods (Figure 1). Researchers at UMass have successfully grown chipilin on black plastic laid to 1.83 meters on center, 31 centimeters in the row and 31 centimeters in-between two rows which gives a density of 29,000 plants ha^{-1}. Transplants are widely used due the low germination in chipilin seed, below 10 percent, and the short growing season (Mangan, 2012).

Two issues have emerged in the production of this crop in Massachusetts. One is the presence of potato leafhopper (*Empoasca fabae*), which produces significant damage to the leaves. For the control a combination of PyGanic®, a botanical insecticide derived from *Chrysanthemums spp* (Griffin, 2012), with the use of synthetic row cover to exclude this insect has shown to be effective. The other issue that farmers are facing is the large amount of nitrogen fertilizer that needs to be applied in order to maintain good quality in leaves. In research trials at the UMass Research farm (South Deerfield, Massachusetts), as much as 300 kg·ha^{-1} has been applied over the course of the season.

As a legume, chipilín has the capacity to fix nitrogen (NifTAL and FAO, 1984) through a symbiotic relationship with bacterial strains of the genus *Rhizobium* (Isidoro and Messier, 2009). Currently farmers in Massachusetts are not using *Rhizobia* since there is not reliable information to use with chipilin and the effect on supplying nitrogen to the plant.

Nitrogen is one of the mayor nutrients needed in the production of food and feed around the world and is the element that limits the quantity and quality of food crops that any other essential element (Prud'homme, 2005). After carbon, nitrogen constitutes the

largest element in plants ranging from 1 to 5 percent of total plant dry matter, and is an essential constituent of proteins, nucleic acids, chlorophyll, co-enzymes, phytohormones and secondary metabolisms (Marschener, 2012).

The amount of nitrogen required for optimum crop growth can be satisfied with organic and inorganic nitrogen sources (Näsholm, et. al., 2009). Inorganic nitrogen sources are manufactured fertilizers, constituting the most important source of nitrogen for commercial-produced plants (Havlin, *et al.,* 2005). Organic sources of nitrogen originated or are synthetized by organisms and include crop residues, animal manure, human wastes and biological fixed nitrogen (Näsholm, et. al., 2009).

Several methods have been developed to determine the amount of nitrogen present in plants in order to provide adequate nitrogen applications. One method is the use of Mi-nolta ® SPAD meter. This device is a small hand-held spectrometer, which measures light absorbed by single, leaves providing a non destructive estimate of plant chlorophyll and nitrogen status (Postgate, 1982.). Vos and Bom (1993) reported the first use of the SPAD meter for agricultural purposes and found in potato using the SPAD meter (r^2=0.95). The SPAD meter has the potential to provide an easy and inexpensive way of estimating nitrogen concentrations, but it has to be calibrated according to the variety and growth stage of each plant, as well as the measurement point and position of the leaf (Lin, *et al.,* 2010).

Nitrogen is one of the most widely distributed elements in nature (Mengel and Kirkby, 1982) however higher plants cannot metabolize N_2 directly from the atmosphere (Havlin, *et al.,* 2005). This nitrogen is only available to plants that are capable of forming symbiosis with N_2-fixing soil bacteria (Marschener, 2012).

Biological nitrogen fixation is the process by which nitrogen is taken from its natural, relatively inert molecular form (N_2) in the atmosphere and converted into nitrogen compounds (ammonia, nitrate and nitrogen dioxide) (NifTAL and FAO, 1984). This process is performed naturally by a number of different prokaryotes (bacteria, actinobacteria, and certain types of anaerobic bacteria) (Couto, 2008), and depends on bacteria enzymic reduction of N_2 via nitrogenase (Mcneil and Materne, 2007). Some nitrogen-fixing bacteria such as Rhizobium infect the root of leguminous plants such as peas (*Pisum sativum* L.), beans (*Phaseolus vulgaris* L.) and clover (*Trifolium pretense* L.) where they form nodules and establish a mutually beneficial association (Hogg, 2005).

The symbiotic relationship between legume host plant and nodule bacteria is mutually beneficial (Havlin, *et al.,* 2005). The infection of a host plant with *Rhizobium* bacteria strains starts with the penetration of the bacteria into a root hair cell (Mengel and Kirkby, 1982). The first stage of the interaction occurs soon after the germination of the legume seed in soil containing a *Rhizobium* species capable of infecting it (Beringer, *et al,.* 1982), justifying the value of the bacteria to be present at the site of root development (Sangakkara, 1989). The plant respond to this infection is by forming tumor-like structures, called nodules, on the root surface.

Through nitrogen fixation, the legume provides organic acids as a carbon source for the bacteria (Mcneil and Materne, 2007), and the specialized Rhizobia bacteris inside the nodule absorb N_2 and convert it to NH_4, using the enzyme nitrogenase and energy from the transformation of ATP to ADP to break the strong triple bond in N_2 (Havlin, *et al.,* 2005). However, this process is highly energy demanding, it is estimated that at least 16 molecules of ATP are required for the reduction of each molecule of di-nitrogen (Atkins, 1984).

Leguminous species are susceptible to nodulation by specific strain of rhizobia. The concept of a cross-inoculation is used for a group of plants that have a specific preference for a specific strain of Rhizobia (NifTAL and FAO, 1984). In order to match symbionts prudently for maximum nitrogen symbiosis, a compatible combination of legume and Rhizobia strain is required (Denison, 1999).

Several environmental factors can affect the formation of nodules and the fixation of nitrogen; these include insufficient soil moisture, insufficient oxygen supply in the soil, nutrient deficiencies or imbalances (Ohyama, 2008) and low soil pH (Mengel and Kirkby, 1982). The quantity of N_2 assimilated by Rhizobium bacteria also depends to a large extent of nutritional conditions. For example nodulation (size and number) is favored by Ca^{2+}, whereas high concentrations (>1mM) of nitrate, nitrite, NH_4 and urea restrict nodulation (Mengel and Kirkby, 1982). McNeil and LaRue, (1984) reported that additional doses of applied nitrogen could inhibit fixation and lead to loss of fixation capacity and yield reductions. In addition, an assay conducted with lentils (*Lens culinaris* L.) demonstrated that fixation rate decreases significatively with the increase of available

soil nitrogen (Bremer et al., 1988). Other important barrier for nitrogen fixation efficiency is the lack of sufficient Rhizobia in the soil to form the maximum amount of nodules on host plants (Kumar, 2012). Additionally low population of compatible Rhizobia, or dominance of inefficient strains of indigenous rhizobia in the soil may decrease nitrogen fixation activity (Ohyama, 2008).

The overall negative environmental impact and the increasing expense of nitrogen fertilizers gives nitrogen fixed legume crops an important advantage for being independent of soil nitrogen (Mcneil and Materne, 2007), where legumes hold a position of special significance as a renewable source of nitrogen for agricultural soils (Peoples, *et al.,* 2009). It is estimated that about 20 percent of nitrogen supplied to commercial crops in the United States is from legumes and crop residues and an approximate estimation of the annual biological N_2 fixation worldwide suggest a range from 130 to 180 $X10^6$ metric tons in 2001 (Havlin, *et al.,* 2005).

In order to benefit farmers who growth chipilin, the following objectives for this research have been established:

- To determinate the nitrogen requirements for chipilín (*Crotalaria longirostrata* Hook. & Arn.).
- To quantify the capacity of select Rhizobia stains to infect and provide nitrogen to chipilín.
- To determinate the amount of nitrogen supplied to chipilín by different strains of Rhizobia.

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CHAPTER 2

YIELD RESPONSE OF CHIPILIN (*Crotalaria longirostrata* Hook. & Arn.)

TO NITROGEN FERTILIZATION IN FIELD CONDITIONS IN

MASSACHUSETTS

2.1. Introduction

Chipilin (*Crotalaria Longirostrata* Hook & Arn), also know as chepil, tcap-in or chop, constitutes an important ingredient in traditional dishes in countries of Central America and some parts of southern Mexico. Chipilin is a member of the Leguminosae family, subfamily Papilionoidea, and the tribe Genisteae (Miller, 1967). It is an herbaceous to woody perennial plant and the most edible specie in the *Crotalaria* genus (FAO, 2012).

The state of Massachusetts, as the country as a whole, has experienced an increasing number of immigrants, especially from the Latin American countries. Latinos are the largest ethnic minority in Massachusetts with 8% of the population (Mangan, *et al.,* 2008). Since 2007, researchers at the University of Massachusetts Amherst have been evaluating chipilin as a potential crop for markets in New England and for production by farmers. With an estimated weekly production of 800 kg·ha^{-1} and a wholesale price of $8.83 kg^{-1}, chipilin has been successfully produced by commercial farmers in Massachusetts. In order to achieve acceptable marketable leaf quality as much as 300 kg·ha^{-1} of nitrogen has been necessary to apply over the course of the season.

Nitrogen is an element needed for plant growth and an essential component of amino acids and proteins in the plant, representing the element taken up in the largest amount by plants. It is part of living cells and chlorophyll, having an essential role in metabolic processes involved in the synthesis and transfer of energy (Marschner et. al., 2003). Widely known as one of the most important limiting factors in the production of food, it has a major effect in the production and profitability in agriculture (Skalsky, *et al.,* 2008).

It is estimated that 200 million tons of nitrogen are added annually to the soil by nitrogen fixing organisms (Hunt, 1996), placing biological nitrogen fixation as an essential process to provide nitrogen for agricultural crops. As a legume, chipilin can be infected by Rhizobia strains (Bisson and Mason, 2010*)* as part of a symbiotic relationship to provide nitrogen to the plant in return for carbohydrates (NifTAL and FAO, 1984).

Plants that form a symbiosis with nitrogen-fixing bacteria have three potential sources of nitrogen: nitrogen fixed from their bacterial symbiont, organic nitrogen sources and from inorganic nitrogen (Na ̈sholm *et al.,* 1998). It is known that nitrogen soil availability can depress symbiotic nitrogen fixation; however, the impact on nodulation due to high nitrogen can vary tremendously (Markham and Zekvel, 2007). As an example, for alfalfa (*Medicago sativa*) nitrogen fertilizer application of 40 and 80 kg·ha^{-1} decreased nitrogen fixation in regrowing plants (Hanaway, *et al.,* 1992).

With the overall goal to evaluate the response of chipilin to soil applied nitrogen, a preliminary trial to estimate the response of chipilin to six nitrogen rates in field conditions was developed in 2011 at the UMass Research Farm in Deerfield, MA.

2.2 Materials and Methods

Under field conditions, a trial was conducted in the summer of 2011 to determine the response of chipilin to soil nitrogen applications. This experiment was implemented at the UMass Research Farm in South Deerfield, Massachusetts. The soil is an Occum fine sandy loam (coarse-loamy, mixed, mesic Fluventic Dystrudept) with a pH of 6.5, 2.4 percent of organic matter, 10 ppm of P_2O_5, 53 ppm of K, 556 ppm of Ca, 65 ppm of Mg and 91.23 ppm of NO_3. Six nitrogen rates (kg·ha^{-1}): 40, 80, 120, 160, 200, 240 were used as treatments. The experiment was set up in a complete randomized block experimental design with four replications.

Chipilin seedlings were produced with seed provided by CENTA (Centro Nacional de Tecnologia Agropecuaria y Forestal) in El Salvador. Chipilin seedlings were started in plastic trays (27.94 x 54.3 centimeters) using Pro-Mix BX-2® (Premier Horticulture Quebec, Canada) as medium and vermiculite placed on top after planting. Approximately twenty days after seeding, the plants were transplanted in 72 square cells plastic trays using the same medium. Harvest Farm (Whately, MA) produced the transplants under greenhouse conditions kept at ambient light, and a temperature of 21 ° C during the day and 16 ° C at night.

In the field, degradable black mulch (121.93 centimeters width and 0.6 millimeters thickness, BioTelo®) was laid every 1.83 meters on raised beds of 10 centimeters. Two rows of plants were transplanted per row of plastic with 31 centimeters in the row and 31 centimeters in-between each row giving a density of 29,000 plants per hectare. Each plot consisted of 12 plants.

Phosphorus and potassium fertilizers were applied through the drip irrigation according to the recommendation for collards, a leafy green that is also grown for multiples harvests (New England Vegetable Management Guide 2011-2012). Nitrogen was applied weekly by hand in ten equal amounts over the course of the experiment using calcium nitrate ($CaNO_3$) as a nitrogen source. Water was provided through drip irrigation according to soil tensiometers (Irometer Co Riverside CA), placed at 38 and 76 centimeters in the soil.

Plants were harvested when they reach marketable size of 20 to 25 centimeters in height. At each harvest the plant height was taken for five plants and the shoot fresh weight and dry weight of ten plants in the plot were taken.

Using the Statistical Analysis System (SAS) program 9.3, the data were submitted to analysis of variance (F test) and orthogonal polynomial comparison procedures. Regression (linear, quadratic and cubic) significance and curves were defined for the nitrogen effect.

2.3 Results

For the two dependent variables chipilin plant length and fresh weight, only the results for fresh weight were statistically significant (Table 2.1). Fresh weight went from 6,293 kg·ha^{-1} with the application of 40 kg·ha^{-1} to 5,393 kg·ha^{-1} with the highest application of 240 kg·ha^{-1}. The highest fresh weight response was 6,808 kg·ha^{-1}, obtained with the application of 120 kg·ha^{-1} of nitrogen, and the lowest was with the application of 240 kg·ha^{-1} of nitrogen (5,393 kg·ha^{-1}).

Table 2.1 Analysis of variance results for plant length and plant fresh weight for chipilin grown at the UMass Research Farm in Deerfield MA in 2011.

	Plant length	Fresh weight
	(*cm*)	(*kg·ha⁻¹*)
Nitrogen rates Ca NO$_3$ (*kg·ha⁻¹*)		
40	13.5	6,293
80	13.6	6,265
120	13.8	6,808
160	13.8	6,610
200	13.3	6,158
240	12.5	5,393
Significance	NS	**
Nitrogen rates (N) [Z]	NS	**Q

[NS,*] Nonsignificant or significant at $P \leq 0.001$.
[Z] Q represents a significant quadratic relationship between nitrogen treatment and the measured parameter.

A highly significant quadratic relationship was the best fit for fresh weight with respect to nitrogen rates, which accounted for 89.3% of the sum square (Figure 2.1). Fresh weight increased from 40 to 120 kg·ha⁻¹ of nitrogen and then it decreased from 120 kg·ha⁻¹ of nitrogen to 240 kg·ha⁻¹.
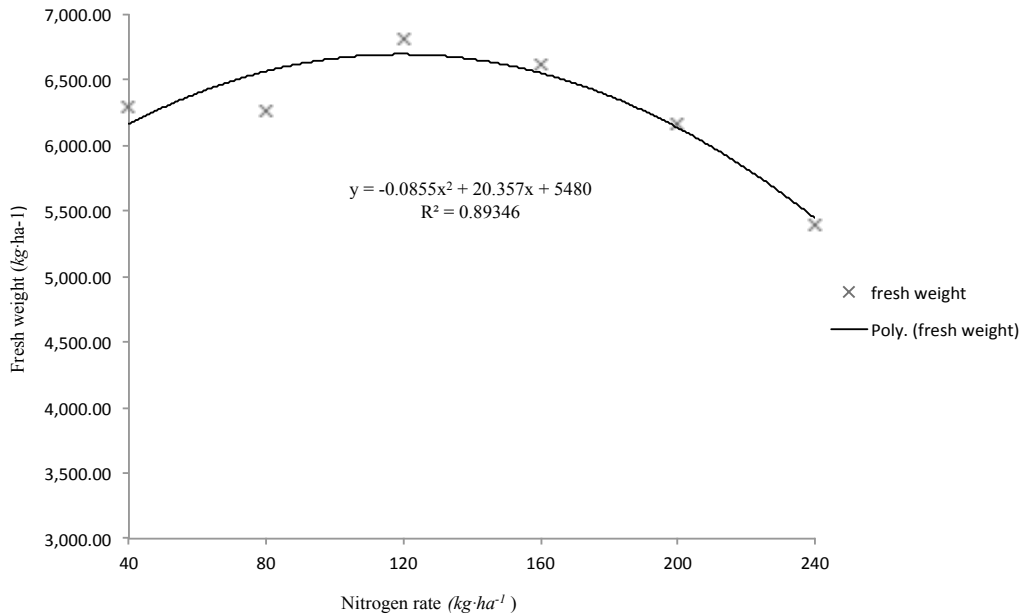
Figure 2.1 Response of fresh weight of chipilin to six nitrogen rates grown at the UMass Research Farm, South Deerfield, MA in 2011.

2.4 Discussion

The expectation was that chipilin yield would increase, or at least remain stable with the application of higher nitrogen rates. However because of the decrease on chipilin yield with applications above 120 kg·ha^{-1} (Figure 2.1), it is reasonable to assume that with an accumulated yield of 6,808 kg·ha^{-1} with nitrogen fertilizer application of 120 kg·ha^{-1}, nitrogen applications above this amount are not needed for chipilin production in Massachusetts. The decrease in yield in addition to 120 kg·ha^{-1} could be due the use of drip irrigation and plastic mulch, which may have reduced nitrogen leaching and thus the soluble salt concentration increased to toxic levels above 120 kg·ha^{-1}.

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CHAPTER 3

FIELD EVALUATION OF THE NITROGEN NEEDS AND EFFICIENCY OF

RHIZOBIA STRAINS FOR CHIPILIN (*Crotalaria longirostrata* Hook. & Arn.)

3.1. Introduction

Chipilin (*Crotalaria longirostrata* Hook. & Arn) is a leafy vegetable used in the preparation of traditional dishes in Central America and parts of the Southern Mexico. Due to the increasing immigrant population from these countries in the Northeastern United States, in 2007 researchers at the University of Massachusetts began to evaluate production practices to produce chipilin in Massachusetts to meet the demand for this popular vegetable. Production practices were established that led to yields in excess of 11, 000 kg·ha^{-1} at the UMass Research Farm in Deerfield MA, and commercial farmers began growing chipilin on their farms in 2009. Preliminary trails found that in order to achieve acceptable leaf quality farmers have to apply as much as 300 kg·ha^{-1} of nitrogen, which is more than twice the amount recommended for most vegetables crops grown in New England (New England Vegetable Management Guide, 2011).

As a legume, chipilin can be infected by strains of Rhizobia (Bisson and Mason, 2010*)* as part of a symbiotic relationship to provide nitrogen to the plant in return for carbohydrates (FAO and NifTAL, 1984). Thus Rhizobia strains represent a sustainable nitrogen source for legumes. For instance, the nitrogen fixed for alfalfa in the United States contributes between 120 and 250 kg·ha^{-1} (Zhu, *et al.,* 1998). Heggo and Barakah (2004) found that between 58 to 83% of the nitrogen required for alfalfa growth is

provided through inoculation with the *R. meliloti* strain, inducing to a significant increase in nodules number on the root system over the control. In mung bean (*Vigna radiata* L.), Kumar, *et al.,* (2012) determined that inoculation with *Rhizobium sp.* strains significantly increase plant growth, number of pods, seed weight, and both grain and straw yield. Similar results were found with the inoculation of *Rhizobium sp.* strains on growth parameters of common beans (*Phaseolus vulgaris* L.), as well as seed protein content (Yadegari and Asadi, 2008).

Chipilin has been observed with live nodules on its roots in El Salvador (Frank Mangan, personal communication, January 20, 2011); however, infection with *Rhizobium* strains has not been detected on chipilin roots under field conditions in New England. Bisson and Mason (2010), in a greenhouse evaluation of Rhizobia strains for chipilin found that three Rhizobia strains; *Bradyrhizobium* USDA 3456, *Bradyrhizobium* PNL0i-Brady and *Bradyrhizobium* USDA 3384, all infect chipilin. They also found that chipilin inoculated with Rhizobia strain *Bradyrhizobium* USDA 3456 had the highest nodule weight compared to the other two strains and the control. Isidoro and Messier (2009), evaluated five strains of Rhizobia on chipilin: *Bradyrhizobium* USDA 3384, 2376 and 3456, *Bradyrhizobium sp*. and a commercial product called Alice for cowpea. They found that all five strains tested positive for host compatibility at low nitrogen levels. They also identified *Bradyrhizobium* USDA 3456 as the most efficient strain, which formed nodules in 100% of plants, had the highest crop yield and a high health score compared with the other strains and the control.

Nitrogen fixation efficiency is affected by several factors, directly influencing the amount of nitrogen that can be supplied to leguminous plant by Rhizobia strains. One of these factors is the application of large amounts of nitrogen fertilizer to the soil (Trabulsi and Abed, 1986.), which negative affects by reducing nodulation and nitrogenase activity (Heggo and Barakah, 2004). According to Zhu, *et al.,* (1996), nitrogen application in access of 100 kg·ha^{-1} on leguminous species can negatively affect root nodule formation, nodule size and weight. Trabulsi and Abed (1986), found that the application of 120 kg·ha^{-1} of soil applied nitrogen substantially suppressed root nodulation of alfalfa and soybean. Also the fact that certain plants show a specific preference for certain rhizobia strains and vice versa, the "cross inoculation" concept, (FAO and NifTAL, 1984.); makes necessary the evaluation of leguminous species response to the interaction of Rhizobia strains inoculum with nitrogen soil availability. These findings justify the evaluation of the interaction between potential inoculants Rhizobia strains for chipilin and the nitrogen fertilizer applications.

This work was conducted to evaluate the effect of the nitrogen fertilization and Rhizobia inoculations on chipilin yield and quality under field cultivation conditions in the state of Massachusetts, USA in 2012.

3.2 Materials and Methods

A field evaluation was conducted in 2012 to assess the effect of three Rhizobia strains and eight nitrogen rates on the growth and quality of chipilin. This evaluation was implemented at the UMass Research Farm in South Deerfield, Massachusetts. The soil is an Occum fine sandy loam (coarse-loamy, mixed, mesic Fluventic Dystrudept), with a

pH of 6.6, 3.0 percent of organic matter, 22 ppm of P_2O_5 and 91 ppm of K. The eight

nitrogen rates were (kg·ha^{-1}): 0, 40, 80, 120, 160, 200, 240 and 280. The four Rhizobia

strains treatments were: *Bradyrhizobium sp. (Vigna), Bradyrhizobium* USDA 3384,

Rhizobium leguminosarum biovar and no inoculation (control). The experiment was set

up as a factorial design as a randomized complete block with five replications.

Degradable black mulch film (121.93 centimeters width and 0.6 millimeters

thickness, BioTelo®) was laid every 1.83 meters on raised beds of 10 centimeters. Two

rows of plants were transplanted per row of plastic with 31 centimeters in the row and 31

centimeters in-between each row giving a plant population of 29,000 plants per hectare.

Each plot consisted of 16 plants.

Chipilin seedlings were produced with seed provided by CENTA (Centro

Nacional de Tecnologia Agropecuaria y Forestal) in El Salvador. Since seed germination

was lower than 10 percent, seeds were started in plastic starter trays (27.94 x 54.3

centimeters) using Pro-Mix BX-2® (Premier Horticulture Quebec, Canada) as a medium

and vermiculite placed on top after planting. After twenty days the plants were

transplanted in 72 square cells plastic trays (27.94 x 53.9 centimeters) using the same

medium. The transplants were produced at Harvest Farm (Whately, MA) under

greenhouse conditions with ambient lights, kept at 14 hours light, and a temperature of

21º C during the day and 16 º C at night.

Bradyrhizobium USDA 3384 was chosen based on the results of Isidoro and Messier (2009). These strains were grown for seven days in liquid Modified Arabinose Gluconate (MAG- appendix Number 1), in an adjusted pH of 6.6. In a glass test tube (20 x 150mm) with 100 ml of liquid MAG medium the bacteria was collocated with an inoculation loop holder (4mm Ø). The test tube was then placed to reconstitute culture on a Gyratory Shaker for growth to 200-RPM (revolutions per minute) to ensure proper aeration. When sufficient growth was observed, it was placed on petri dishes (100x15mm) with solid MAG medium in dilutions of 1:1, 1:10, 1:100 and 1:1000 and kept at 30 ºC. The bacteria strain was streaked on solid MAG media after five days. Singles colonies were observed after seven days on plates streaked for isolation while maintaining the temperature at 30 ºC. All transfer work performed under a Laminar Flow Hood. From the bacteria growth in the petri dishes, a solution was made in a minimum concentration of $2x10^8$. Ten milliliters of the bacteria dissolution was applied with a 5 ml plastic syringe to the chipilin roots in flats within 72 square cells plastic trays.

The other two strains were *Bradyrhizobium sp. (Vigna)* (N-DURE® inoculant for cowpea (*Vigna unguiculata* L.)) and *Rhizobium leguminosarum biovar* (N-DURE® inoculant for beans (*Phaseolus spp*. L.)), which were inoculated through the commercial products from INTX microbials-llc, Kentland, Indiana. Such strains were chosen based on the recommendation of researchers at CENTA who have been testing inoculants for chipilin. The dilution was made with water and 10 ml was applied to the chipilin roots as described above for the others strains. Both strains were applied with a minimum bacteria concentration of $2x10^8$ CFU.

Phosphorus and potassium fertilizers were applied through the drip irrigation according to the recommendation for collards, a leafy green that is also grown for multiples harvests (New England Vegetable Management Guide 2011-2012). Nitrogen was applied weekly by hand eight times over the course of the trial, using ammonium nitrate ($NH_4 NO_3$) as nitrogen source. Thirty percent of the total nitrogen was applied weekly during the first three weeks of the experiment; and the remaining 70 percent in five applications of 14 percent of the total for each rate. Water was provided through drip irrigation according to soil tensiometers (Irometer Co Riverside CA), placed at 38 and 76 centimeters below the soil surface.

Plants were harvested when they reached marketable size (20 to 25 centimeters height). For each harvest qualitative and quantitative data were taken from a sample of four plants per plot. Qualitative data consisted of color, vigor and uniformity using a rating scale from 1 to 5. Quantitative data consisted of SPAD readings (Minolta SPAD-502), plant height (centimeters) from a sample of four plants, shoot fresh weight ($gr \cdot plant^{-1}$) and shoot dry weight ($gr \cdot plant^{-1}$) were taken of 12 plants in the plot. Determination of total nitrogen content in plants ($mg \cdot liter^{-1}$) also was assessed through Vario Max ® analysis system (Vario Max N, CNS, CHN- Operation Manual). At the end of the experiment roots system from four plants per plot were dug up, in an area of 35 cm^2 around the plant, and carefully washed. Inoculation incidence was determined for four plants per plot. Randomly chosen, one plant was taken to the oven, and when it had constant weight, nodules number per plant and nodules weigh were determined.

The Statistical Analysis System (SAS) program 9.3 was used for the statistic analysis. Data were submitted to analysis of variance (test F), followed by Duncan's new multiple range test for mean comparation of the Rhizobia strains effects. In addition, for nitrogen rates effect; orthogonal polynomial comparison and regression (linear, quadratic and cubic) significance and curves were defined.

3.3 Results

Table 3.1 shows the ANOVA results for the two main effects in the experiment, nitrogen rates and Rhizobia strains, for 12 dependent variables and their interaction. For nitrogen, all the dependent variables were highly significant ($P \leq 0.001$) except inoculation percentage, nodule number and nodule weight which were not statistically significant (Table 3.1).

Table 3.1. Statistical differences of the two main effects, nitrogen rates and Rhizobia strain, and their interaction for 12 dependent variables for an experiment with chipilin grown at the UMass Research Farm in Deerfield MA in 2012.

| | | Main effects | | |
	Dependent variable	Nitrogen[1] (N)	Rhizobia strain[2] (R)	(R) X (N)
Quantitative parameters	Fresh weight	**	*	NS
	Dry weight	**	*	NS
	SPAD readings	**	*	NS
	Plant length	**	**	NS
	Total nitrogen	**	NS	NS
	Inoculation percentage	NS	*	NS
	Nodule number	NS	*	NS
	Nodule weight	NS	NS	NS
Qualitative parameters	Color	**	NS	NS
	Vigor	**	NS	NS
	Uniformity	**	NS	*

[NS,*,**] Nonsignificant or significant at $P \leq 0.05$ or 0.001 respectively.
[1] Data obtained for the overall nitrogen rates response.
[2] Data obtained for the overall Rhizobia strain inoculation response.

For Rhizobia, only plant length was highly significant while fresh weight, dry weight, SPAD readings, inoculation percentage and nodule number were significant ($P \leq 0.05$). Total nitrogen, nodules weight, color, vigor and uniformity were not statistically significant. No significant interaction of the two main effects was determined for any of the dependent variables, except for uniformity.

Chipilin fresh weigh and dry weight were lower for plants inoculated with *Bradyrhizobium* USDA 3384 compared to *Bradyrhizobium sp. (Vigna)*, *Rhizobium leguminosarum biovar* and the control (Table 3.2). The lowest value response for plant length was 36.4 cm, also obtained with *Bradyrhizobium* USDA 3384.

For SPAD readings taken on chipilin leaves, *Bradyrhizobium* USDA 3384 had the highest reading at 47. Although that *Bradyrhizobium* USDA 3384 obtained the highest SPAD values, no significant difference was found for total nitrogen compared with the other two strains and the control. The highest inoculation percent by Rhizobia was found with *Bradyrhizobium* USDA 3384 and *Bradyrhizobium sp. (Vigna)*, with 98.7% and 98.1% respectively. Despite the fact that chipilin plants in the control treatment were not inoculated in the greenhouse, 92.5% of the plants had nodules.

Table 3.2. Fresh weight yield (FW), dry weight yield (DW), SPAD readings (SPAD), total nitrogen determination (TN), plant length (PL), total inoculation percentage (IP), nodule number per plant (NN) and weight per nodule (NW) means for three Rhizobia strains and a control in chipilin grown at the UMass Research Farm in Deerfield MA in 2012. The values are an average of eight N rates.

	FW	DW	SPAD[1]	TN	PL	IP	NN	NW
	$(kg \cdot ha^{-1})$	$(kg \cdot ha^{-1})$		$(mg \cdot l^{-1})$	$(cm\ plant^{-1})$	(%)	$(plant^{-1})$	$(mg \cdot nodule^{-1})$
Rhizobium strain								
Control	6,674.4 a	934.8 a	46.6 ab	5.0	38.1 a	92.5 b	14.3 a	0.8
Bradyrhizobium sp. (Vigna)	6,646.4 a	941.2 a	46.0 b	5.0	37.8 a	98.1 a	15.2 a	0.8
Bradyrhizobium USDA 3384	6,077.2 b	858.0 b	47.0 a	5.1	36.4 b	98.7 a	9.0 b	0.4
Rhizobium leguminosarum biovar	6,832.4 a	956.4 a	46.0 b	5.1	38.3 a	97.4 ab	15.0 a	1.7
Significance	*	*	*	NS	**	*	*	NS

[NS,*,**] Nonsignificant or significant at $P \leq 0.05$ or 0.001 respectively.
Mean separation in columns by Duncan's new multiple range test (P=0.05)
[1] SPAD plant leave measurement, Minolta Co. Ltd.

The lowest number of nodules per plant was nine, found with the Rhizobia strain *Bradyrhizobium* USDA 3384 inoculation which was statistically lower than the number of nodules found on roots in the control, *Rhizobium leguminosarum biovar* and *Bradyrhizobium sp. (Vigna)*, with 14.3, 15.0 and 15.2 nodules per plant respectively. For this experiment no statistical significance in nodule weight was found among Rhizobia treatments.

For the qualitative variables leaf color, plant vigor and uniformity no statistical differences among the three Rhizobia strains and the control was found (Figure 3.3).

Table 3.3. Mean comparison for qualitative variables leaf color, plant vigor and uniformity of chipilin grown with inoculation of three Rhizobia strains and a control at the UMass Research Farm in Deerfield MA in 2012. The values are an average of eight N rates.

	Color	Vigor	Uniformity
	(1 to 5)	*(1 to 5)*	*(1 to 5)*
Rhizobium strain			
Control	3.1	3.2	2.9
Bradyrhizobium sp. (Vigna)	3.2	3.3	3.1
Bradyrhizobium USDA 3384	3.1	3.2	3.0
Rhizobium leguminosarum biovar	3.1	3.3	3.0
Significance	NS	NS	NS

[NS] Non significant.

Mean separation in columns by Duncan's new multiple range test (*P*=0.05)

[1] Values are based on a qualitative scale where 1 corresponds to the lowest and 5 to the highest response for each dependent variable.

Leaf color, plant vigor and uniformity also had a highly significant linear, quadratic and cubic response to nitrogen rates (Table 3.4).

Table 3.4. Leaf color, plant vigor and uniformity of chipilin grown under eight nitrogen rates at the UMass Research Farm in Deerfield MA, 2012. The values are an average of three Rhizobia strains and a control.

	Color[1]	Vigor[1]	Uniformity[1]
	(1 to 5)	*(1 to 5)*	*(1 to 5)*
Nitrogen rate $NH_4 NO_3$ *(kg·ha^{-1})*			
0	2.3	2.5	2.3
40	2.9	3.0	2.9
80	3.3	3.2	2.9
120	3.2	3.5	3.2
160	3.4	3.4	3.2
200	3.3	3.3	3.0
240	3.4	3.6	3.2
280	3.5	3.5	3.3
Significance	**	**	**
Nitrogen treatments (N) [z]	**L, **Q, **C	**L, **Q, **C	**L, **Q, **C

[NS,**] Nonsignificant or significant at $P \leq 0.01$ respectively.

Mean separation in columns by Duncan's new multiple range test (*P*=0.05)

[1] Values are based on qualitative scale where 1 corresponds to the worst and 5 to the best response for each dependent variable.

Chipilin fresh weight, dry weight, plant length, and the total nitrogen for the main effect nitrogen were highly significant (Table 3.1), and also had a highly significant linear, quadratic and cubic response to nitrogen rates (Table 3.4). With no fertilizer nitrogen applied, chipilin produced 4,564 kg·ha^{-1} of fresh weight, and with 280 kg·ha^{-1} of nitrogen applied the fresh weight was increased to 7,229 kg·ha^{-1}. The dry weight had a similar response; 662 kg·ha^{-1} of chipilin were accumulated without any nitrogen applied, and 1,032 kg·ha^{-1} with 280 kg·ha^{-1} of nitrogen. Plant length and total nitrogen, which also had a highly significant linear, quadratic and cubic response, plant length ranged from 33.8 to 38.7 cm and total nitrogen ranged from 4.4 to 5.3 mg·l^{-1}. There was no statistical significance for nodules number per plant and nodules weight.

Table 3.5. Fresh weight (FW), dry weight (DW), leaf SPAD readings (SPAD), plant length (PL), plant total nitrogen (TN), total root inoculation percentage (IP), nodule number per plant (NN) and nodule weight (NW) of chipilin grown under eight nitrogen rates at the UMass Research Farm in Deerfield MA, 2012. The values are an average of three Rhizobia strains and a control.

	FW	DW	SPAD	PL	TN	IP	NN	NW
	$(kg \cdot ha^{-1})$	$(kg \cdot ha^{-1})$		$(cm \, plant^{-1})$	$(mg \cdot l^{-1})$	(%)	$(plant^{-1})$	$(mg \cdot nodule^{-1})$
Nitrogen rate								
$NH_4 \, NO_3 \, (kg \cdot ha^{-1})$								
0	4,564	661	44.1	33.8	4.4	93.7	16.4	1.9
40	5,892	838	45.5	36.9	4.9	96.2	14.1	1.4
80	6,974	982	45.9	39.1	5.0	100.0	16.7	0.6
120	6,950	982	46.3	38.6	5.1	98.7	13.2	1.9
160	7,067	982	46.8	38.0	5.2	96.0	12.5	0.5
200	6,870	941	46.8	37.5	5.2	97.5	12.0	0.4
240	6,913	960	47.2	38.6	5.3	97.5	11.0	0.5
280	7,229	1031	48.1	38.7	5.3	93.7	12.8	0.4
Significance	**	**	**	**	**	NS	NS	NS
N treatments (N)[z]	**L, **Q, **C	**L, **Q, **C	**L, **C	**L, **Q, **C	**L, **Q, **C	NS	NS	NS

[NS,**] Nonsignificant or significant at $P \leq 0.01$.
[z] L, Q, C represents a significant linear, quadratic or cubic relationship respectively between nitrogen treatment and he measured parameter.

3.4 Discussion

It should be noted that the highest fresh weight response, 7,229.2 kg·ha^{-1} obtained

with the overall application of 280 kg·ha^{-1} of nitrogen (Table 3.4) was not as high as the

yield reported by commercial farmers in Massachusetts, which can exceed

11,000 kg·ha^{-1}. This is most likely due the fact that this experiment was started as much

as five weeks later than commercial farmers normally plant chipilin. Furthermore, it is

noteworthy to point out that seed produced by chipilin vary widely in genetic traits

because it is an open-pollinated specie (Morton, 1994). Additionally, the seed used in this

project was not produced from an established breeding program, which might have

produced a high viability in seed germination and variability of plant growth. The low

fresh and dry weight as well as plant length obtained with *Bradyrhizobium* USDA 3384

inoculation (Table 3.2) could be a result of the inoculation with an inefficient strain for

chipilin. There is a range in the energy cost of nitrogen fixation compared to nitrate

assimilation (Burdas, 2002), and it is possible that the *Bradyrhizobium* USDA 3384

physiologically assimilated nitrogen less costly from soil as ammonium and nitrate than

through nitrogen fixation (Postgate, 1982).

For the chipilin plants that were not inoculated, 92.5% of the roots were nodulated

to which three factors can be possible reasons. One of the possible causes may be due to

the fact that many soils can have diverse Rhizobia strains capable of inducing root

nodulation (FAO and NifTAL, 1984) and one of these Rhizobia strains could have

inoculated the chipilin plants in the control treatments. A second factor may be as a

consequence of Rhizobia inoculum present in the seed. And a last reason could be

contamination of the control plants during the inoculation process in setting up the experiment. It is also possible to be the result of a combination of any of the above preceding scenarios.

In a greenhouse experiment implemented to evaluate the effect of Rhizobia nodulation on chipilin growth, nodules were also found on the roots of control plants where no Rhizobia strain was applied (Bisson and Mason, 2010). The fact that the mediumused in this trial was sterile sand and perlite means that contamination could not come from the medium. The authors suggest that this contamination was due the presence of Rhizobia in the seed. Considering that the prevalence and survival of Rhizobia in seed is influenced by environmental factors that lead to low O_2 levels and desiccation (Deaker, *et at.,* 2004), the possibility of a long term presence of Rhizobia inoculum in chipilin needs to be established.

According to Burdass (2002), the increase in nodule number on infected legume roots is closely related with the rate of nitrogenase activity and efficiency of nitrogen fixation. With a lower rate of nodulation, *Bradyrhizobium* USDA 3384 could be due to the fact that it is a lower nitrogen efficiency strain for chipilin. The same author cites that lower rates of nodulation can be related to lower amounts of inoculum introduced to the roots, and a genetic incompatibility of *Bradyrhizobium* USDA 3384 with the inoculated species are possible factors contributing to this response. FAO and NifTAL (1984) reported that there is a relationship between the size of nodules and their effectiveness in fixing nitrogen, with larger nodules being more effective in nitrogen fixation. Without statistical significance in the size of the nodules for any of the strains and the control, it is

not possible to conclude that fixation efficiency for chipilin among the Rhizobia strains due to the size of the nodules.

Results suggest that nitrogen applications above 80 kg·ha^{-1} did not increase chipilin yield (Figure 3.4); however SPAD readings did increase suggesting that the extra nitrogen applied above this rate is more for leaf quality than for higher yield. The yield response had statistically significant linear and quadratic responses due to the leveling off of yield with more than 80 kg·ha^{-1} whereas the SPAD readings had a highly significant linear response and not for quadratic since there was not leveling off of values above 0 kg·ha^{-1}. This suggests that the major effect of nitrogen applications above 80 kg·ha-1 is to enhance the leaf color in the chipilin leaves rather than increased chipilin yield.

Green leaves are an important indicator of chipilin quality. In the case of Rhizobia, total nitrogen was not significant; however, SPAD readings were. *Bradyrhizobium* USDA 3384 had higher SPAD readings than the other two Rhizobia strains, it is speculated that the smaller plants with *Bradyrhizobium* USDA 3384 meant that the nitrogen in the plants, which was not statistically different that the other Rhizobia treatments and the control, was more concentrated in the leaves which caused higher SPAD readings values. Li, et, al (2012), determined that SPAD readings values are highly correlated with leaf nitrogen concentration for potato leaves. In the case of nitrogen, SPAD readings can be used as a tool to assess nitrogen content in chipilin and can perhaps be used as a diagnostic tool by researchers and commercial growers.

In conclusion, Rhizobia strain *Bradyrhizobium* USDA 3384 is not recommended for inoculated chipilin. Furthermore, in future work it will be critical to identify Rhizobia species that infect chipilin in order to know if there are non-indented Rhizobia inoculating treatments. Nitrogen fertilizer application of 80 kg·ha^{-1} appears to be economically appropriated for chipilin production, but without any specific guaranty of obtaining a marketable leaf color and quality.

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CHAPTER 4

GREENHOUSE EVALUATION OF CHIPILIN (*Crotalaria longirostrata* Hook. & Arn.) RESPONSE TO RHIZOBIA INOCULATION

4.1. Introduction

Chipilin (*Crotalaria longirostrata* Hook. & Arn.) is an annual slender shrubby legume (Morton, 1994) widely distributed in altitudes between 1,000 to 2,200 meters above sea level in Central America and southern parts of Mexico (Chizmar, 2009.). It is considered as the *Crotalaria* species most edible of this genus (FAO, 2012), and is highly valued for its young leaves as an important ingredient in traditional dishes in these regions.

The United States has experienced an increasing number of immigrants, especially from Latino American countries. In Massachusetts, the Hispanic community constitutes the largest ethnic minority with 8% of the population (Mangan, *et at.,* 2008). Starting in 2007 researchers at the University of Massachusetts Amherst began evaluating chipilin as a potential crop for markets in New England, and for production by farmers.

With an estimated weekly production of 11,000 kg·ha^{-1} and a wholesale price of $8.83 kg^{-1}, chipilin has been successfully produced in Massachusetts. In order to achieve a marketable leaf quality as much as 300 kg·ha^{-1} of nitrogen has been necessary to apply to chipilin over the course of the season. Nitrogen fertilizer is considered the most expensive input in nearly all crop production systems (JoAnn, 2011.) and the use of high

amounts of fertilizer has a major effect on production decisions and profitability

(Skalsky, et. al., 2008) to which chipilin is not exempt.

Although there is no reliable information on the potential use of Rhizobia to

provide nitrogen to chipilin in commercial production, as a legume it can be infected by

Rhizobia strains (Isidoro and Messier, 2009.). Legumes represent an essential component

of sustainable agricultural systems (Hunt, 1996.). As an example, it is estimated that 58 to

83% of the nitrogen required for alfalfa growth (*Medicago sativa*) can be supplied

through the nitrogen fixation process (Heggo and Barakah, 2004). However, N_2 fixation

is a costly energy process for legumes (Hussein, 1999). Research has demonstrated that

the energy costs of fixation to the host plant may require more energy than the uptake of

the available nitrogen in the soil (Atkins, 1982), especially if the Rhizobia strain is

inefficient for the host legume species (Lyons, *et at.,* 1980).

Biological nitrogen fixation (BNF) has been assessed as one of the most important

processes in agriculture. It has been estimated that annually four times more nitrogen

(200 million tons) is added to the soil through BNF than with inorganic nitrogen sources

(Hunt, 1996). For legumes, BNF requires the development of a symbiotic relationship

between soil bacteria and the plant root. The most common symbionts found in the roots

of legumes crops are bacteria of the genera *Rhizobium* and *Bradyrhizobium* (Hunt, 1996).

Rhizobia infection is an extremely complex process between host and symbiont, in which

legumes have a certain preference for specific strains of Rhizobia and vice versa,

constituting a "cross-inoculation group" (NifTAL and FAO, 1984).

Nitrogen fixation efficiency can be affected by many factors, including temperature, soil moisture, soil salinity, pH, nutrient levels and nitrogen fertilizer applications (Hussein, 1999). NifTAL and FAO (1984) suggests that with nitrogen fertilizer applications, nodules produced by effective strains of Rhizobia remain small and exhibit the same characteristics as those produced by ineffective rhizobia.

Commercial scale host specific Rhizobia strains are frequently developed for new cultivars or varieties of legumes; however when a leguminous crop is introduced to new areas or regions it is often beneficial to evaluate the need for inoculation as well as the response of the specie to specific Rhizobia strains (NifTAL and FAO, 1984).

In order to define the efficiency of four Rhizobia strains for chipilin and their interaction with seven levels of nitrogen, an evaluation was conducted in the greenhouse of the College of Natural Sciences at the University of Massachusetts Amherst in 2012.

4.2 Materials and Methods

Under greenhouse conditions, a trial was conducted to assess the effect of four Rhizobia strains and eight nitrogen rates on the nodulation of chipilin. The experiment was implemented at the College of Natural Sciences greenhouse facilities at the University of Massachusetts Amherst. With 14 hours of light and a temperature of 19ºC during the day and 18ºC at night, the experiment was set up as a randomized complete bock design with five replications. The Rhizobia strains used as treatments were: *Bradyrhizobium* USDA 3384, *Bradyrhizobium* USDA 2370, *Bradyrhizobium sp. (Vigna)* and *Rhizobium leguminosarum biovar*. Nitrogen treatments were provided through

modified 0.5 M Hoagland solution in seven rates: 0, 26.25, 52.5, 105, 157.5, 210 and

262.5 mg·L[1].

Chipilin seedlings were produced with seed provided by CENTA (Centro

Nacional de Tecnologia Agropecuaria y Forestal) in El Salvador. Seeds were started in

starter plastic trays (27.94 x 54.3 centimeters) using Pro-Mix BX-2® (Premier

Horticulture Quebec, Canada) as a medium and vermiculite placed on top after planting.

After twenty-one days the plants were transplanted into 72 square cells plastic trays

(27.94 x 53.9 centimeters) using the same medium. One application of 200 mg N · L[1] was

applied to all the plants twenty-seven days after the seed was sown. The transplants were

produced at a commercial farm (Harvest Farm Whately, MA) under greenhouse

conditions with ambient light and a temperature of 21º C during the day and 16º C at

night.

Rhizobia strains *Bradyrhizobium* USDA 3384 and USDA 2370 were chosen

based on results of Isidoro and Messier (2009). These strains were grown for seven days

in liquid Modified Arabinose Gluconate (MAG- appendix Number 1), in an adjusted pH

of 6.6. In a glass test tube (20x150mm) with 100 ml of liquid MAG medium the bacteria

was transfered with an inoculation loop holder (4mm Ø). The test tube was then placed to

reconstitute the culture on a Gyratory Shaker for growth to 200-RPM (revolutions per

minute) to ensure proper aeration. When sufficient growth was observed, it was placed on

petri dishes (100x15mm) with solid MAG medium in dilutions of 1:1, 1:10, 1:100 and

1:1000 and kept at 30 ºC. After five days, the bacteria strain was streaked on solid MAG

media. Singles colonies were observed after seven days on plates streaked for isolation

while maintaining the temperature at 30 ºC. All transfer work was performed under a Laminar Flow Hood. From the bacteria growth in the petri dishes, a solution was made in a minimum concentration of $2x10^8$. Ten milliliters of the bacteria dissolution was applied with a 5 ml plastic syringe to the chipilin roots in flats in 72 square cells plastic trays.

Bradyrhizobium sp. (Vigna) (N-DURE® inoculant for cowpea (*Vigna unguiculata* L.)) and *Rhizobium leguminosarum biovar* (N-DURE® inoculant for beans (*Phaseolus spp.* L.)) were applied through the use of N-DURE® commercial products from INTX microbials-llc (Kentland, Indiana). These strains were chosen based on the recommendation of researchers at CENTA who have been testing inoculants for chipilin. The dilution was made with water, and 10 ml were applied to the chipilin roots as described above for the other strains. Both strains were also applied with a minimum bacteria concentration of $2x10^8$ CFU. Inoculations of the four strains were performed 53 days after seed was sown. Five days after the inoculation, three plants were transplanted into standard 300 plastic pots (15 centimeters diameter and 21 centimeters deep) using a mix of sieved, washed river sand and perlite in proportion of 1:1. Fourteen days later, plants were thinned to one plant per pot.

For 12 weeks, plants were treated with an application of 0.5 M strength modified Hoagland solution (Hoagland and Arnon, 1950). Nitrogen treatments were provided through $NaNO_3$ in the following concentrations: 0, 26.25, 52.5, 105, 157.5, 210 and 262.5 mg N $\cdot L^1$. In a combination of KH_2PO_4, $MgSO_4$, $CaCl_2$, KCl, and $NaNO_3$, the solution provided all plants with (mg N $\cdot L^1$): 234 potassium (K), 31 phosphorus (P), 48 magnesium (Mg), 64 sulfur (S), 200 calcium (Ca), 525 chloride (Cl), and 0, 44, 87, 173,

259, 345, 432, sodium (Na) respectively for the nitrogen treatments. The nutrient solution was applied once or twice a week, based on the water requirements of plants. The first solution application was made with 420 mg N $\cdot$L^1 as the highest concentration. However due to the presence of salt toxicity symptoms after this application, 262.5 mg N $\cdot$L^1 rather that 420 mg N $\cdot$L^1 concentration was used in subsequent applications.

Fifty-seven days after starting the experiment, the systemic neonicotinoid insecticide Safari® 20 SG for the control of fungus gnat (*Bradysia coprophila* (Linter)) was applied according to the label specifications (Valent U.S.A. Corporation). This pesticide application caused severe phytotoxicity to the plants resulting in a complete plant defoliation making it impossible to measure plant weight or determine the total nitrogen content in leaves. At the end of the experiment, root systems were carefully washed and the inoculation percentage, number of nodule and weight per plant were determined.

The Statistical Analysis System (SAS) program 9.3 was used for the statistic analysis. Data were submitted to analysis of variance (F test), followed by Duncan's new multiple range test for mean comparison of the Rhizobia strains effects. In addition orthogonal polynomial comparison and linear regression significance and curves were defined for nitrogen rates.

4.3 Results

Results of the analysis of variance for the two main effects, nitrogen and Rhizobia and their interaction, are show in Table 4.1. For nitrogen, only nodule number was statistically significant while for Rhizobia strains the inoculation percentage and nodule number per plant were highly significant ($P \leq 0.01$) and nodule weight was significant ($P \leq 0.05$). The interaction of the two main effects, nitrogen and Rhizobia, was only significant for nodules number.

Table 4.1. Statistical differences of the two main effects, nitrogen concentration and Rhizobia strain, and their interaction for total inoculation percentage, nodule number per plant and nodules weight as dependent variables for a greenhouse evaluation with chipilin grown in a greenhouse at UMass Amherst in 2012.

	Main effects		
	Nitrogen[1] (N)	Rhizobia strain[2] (R)	(R) X (N)
Inoculation percentage	NS	**	NS
Number of nodule	**	**	**
Nodule weight	NS	*	NS

[NS,*,**] Nonsignificant or significant at $P \leq 0.05$ or 0.001 respectively.
[1] Data obtained for the overall nitrogen rates response.
[2] Data obtained for the overall Rhizobia strain inoculation response.

Roots inoculated with the strains *Bradyrhizobium* USDA 3384, *Bradyrhizobium* USDA 2370, *Bradyrhizobium sp. (Vigna)* and *Rhizobium leguminosarum biovar* obtained the highest percentage of inoculation at 91.6, 100.0, 96.9 and 98.3 %, respectively, while the lowest inoculation rate was obtained with the control treatment, without any inoculation at 59.6% (Table 4.2).

Chipilin plants inoculated with *Rhizobium leguminosarum biovar* had the highest

number of nodules per plant with 128 nodules. The control with 23 had the lowest

number of nodule. There was no statistical difference among *Bradyrhizobium* USDA

3384 and *Bradyrhizobium* USDA 2370 with 38 and 39 nodules per plant, respectively.

Table 4.2. Mean comparison for total inoculation percentage, number of nodules per plant, nodule weight and total nodule weight per plant of chipilin grown with inoculation of four Rhizobia strains and a control in a greenhouse, UMass Amherst in 2012.The values are an average of eight N rates.

	Inoculation percentage	Number of nodule	Nodule weight	Nodule weight per plant
	(%)		*(mg·nodule[1])*	*(mg·plant[1])*
Rhizobium strain				
Control	59.6 b	23.0 c	3.3 a	75.9 a
Bradyrhizobium USDA 3384	91.9 a	38.0 bc	2.8 a	106.4 a
Bradyrhizobium USDA 2370	100.0 a	39.0 bc	1.6 ab	62.4 ab
Bradyrhizobium sp. (Vigna)	96.9 a	51.0 b	2.5 a	127.5 a
Rhizobium leguminosarum biovar	98.3 a	128.0 a	0.8 b	102.4 b
Significance	**	**	*	*

[*,**] Significant at $P \leq 0.05$ or 0.001 respectively.
Mean separation in columns by Duncan's new multiple range test (P=0.05)

Although chipilin roots inoculated with *Rhizobium leguminosarum biovar* had the

highest number of nodules per plant, the individual nodules were the smallest of the

Rhizobia treatments including the control with a weight of 0.8 mg·nodule[1].

As stated above, only nodule number per plant for the nitrogen rate was

statistically significant ($P \leq 0.01$) (Table 4.1). This significance also produced a highly

significant linear response for number of nodules per plant (Table 4.3) accounting for

81.6% of the sum of squares. Nodule number decreased from 94.9 per plant with no

nitrogen applied to 19.6 nodules at the highest rate 262.5 mg N ·L[1].

Table 4.3. Response for total inoculation percentage, number of nodule per plant, nodule weight and total nodule weight per plant of chipilin grown with seven nitrogen rates through 0.5M modified Hoagland solution and a control in a greenhouse, at UMass Amherst in 2012.The values are an average of five Rhizobia treatments.

	Inoculation percentage	Number of nodule	Nodule weight	Nodule weight
	(%)		(mg·nodule[1])	(mg·plant[1])
Nitrogen (mg ·L[1])				
0-control	91.2	91.0	1.5	136.5
26.25	92.1	105.2	3.4	357.7
52.5	81.2	62.2	2.5	155.5
105	95.6	51.3	1.9	97.47
157.5	93.1	30.5	1.9	57.95
210	92.5	40.3	1.9	76.57
262.5	82.4	19.6	1.8	35.28
Significance [z]	NS	**	NS	NS
Trend (nitrogen) [z]	NS	**L	NS	NS

[NS,**] No significant and significant at $P \leq 0.01$.
[Z] L represents a significant linear relationship respectively between nitrogen treatment and the measured parameter.

With an overall highly significant effect for nodules number to nitrogen effect (Table 41.), the nitrogen effect within Rhizobia strains shows that nodule number in the control treatment and the strain *Rhizobium leguminosarum biovar* deceased with a highly significant linear ($P \leq 0.01$) response, while *Bradyrhizobium sp. (Vigna)* was found to have a significant linear ($P \leq 0.05$) response (Figure 4.1). The response of *Bradyrhizobium* USDA 3384 and *Bradyrhizobium* USDA 2370 inoculations was not significant.
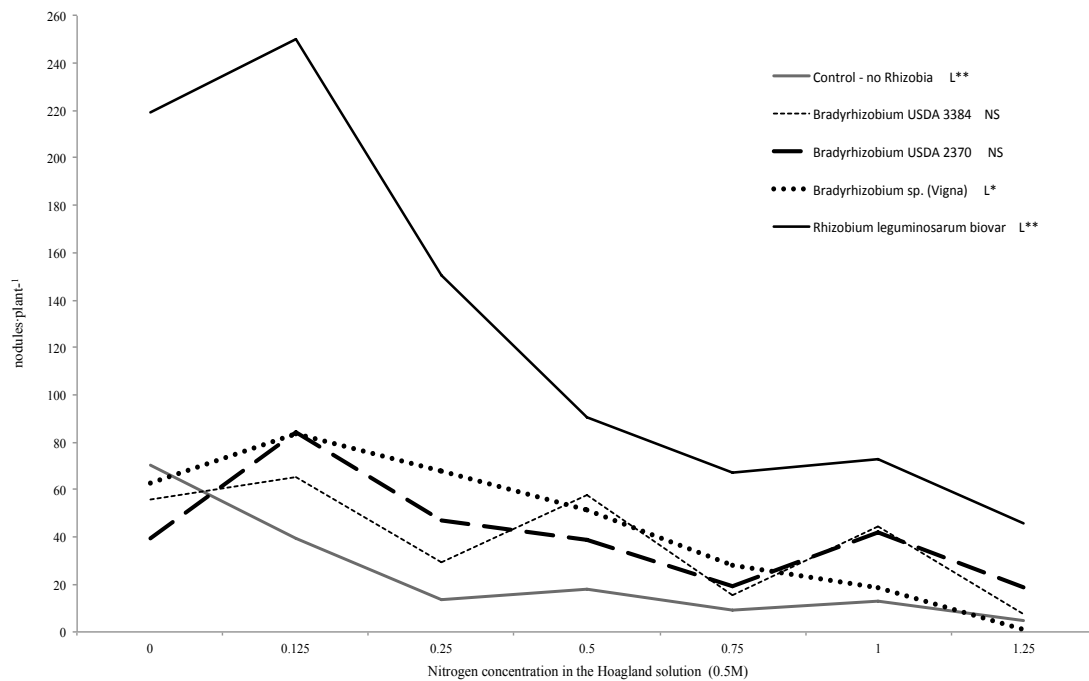
NS*,** No significant, significant at $P \le 0.05$ or 0.001 respectively.
Z L represents a significant linear relationship respectively between nitrogen treatment and the measured parameter.

Figure 4.1 Response for nodule number of inoculated plants with Rhizobia strain to the overall nitrogen concentration in 0.5M modified Hoagland solution of chipilin growth in a greenhouse, UMass Amherst in 2012.

4.4 Discussion

With the highest number of nodules per plant, 128 and inoculation efficiency, 98.3% , *Rhizobium leguminosarum biovar* could be considered as the best strain to inoculate chipilin among the Rhizobia evaluated in this trial (Table 4.2),; however it is not possible to state this definitely without assessing the nitrogen contribution to the chipilin plants inoculated with this Rhizobia strain.

NifTAL and FAO (1984) suggest that with nitrogen fertilizer applications, nodules produced by effective strains of Rhizobia remain small and exhibit the same characteristics as those produced by ineffective Rhizobia. Contrary to these findings, results in this experiment show no significant differences for nodule weight to nitrogen applications (Table 4.3). It is possible that this lack of decrease in nodule size with higher nitrogen rate is due to poor plant growth. As mentioned above, plants were significatively defoliated due the phytotoxicity for the application of Safari® to manage insect pests and this decrease in leaf growth would have also decreased photosynthetic activity causing a reduction in the amount of carbohydrates provided to the nodules and thus potentially reducing nodule growth.

Residual or applied nitrogen levels in soils have been shown to decrease nodulation and nitrogen fixation with increasing levels (Hussein, 1999). This effect is attributed to the inhibition of nitrogenase activity (Arreseigor *et at.,* 1997), root infection (Abdel-Wahab *et at.,* 1996) and nodule development (Imsande, 1986.). Similar results were found on inoculated plants with *Rhizobium leguminosarum biovar*, *Bradyrhizobium sp. (Vigna)* and in the control treatment, where the increase in nitrogen concentration caused a decrease in nodules number per plant (Figure 4.3). This response may be due to the fact that plants have to use less energy in the uptake of nitrogen from the soil (Hussein, 1999) compared to the energy that plant has provide for nitrogen fixation (Atkins, 1982).

Without any inoculation and the use of sand and perlite as media in this experiment, the appearance of nodules on chipilin plants in the control treatments (Table 4.2) suggests the presence of Rhizobia inoculum in the seeds. Another possible cause may be due contamination during the setting up of this experiment. It could be also due to a combination of these two scenarios. Considering that the prevalence and survival of Rhizobia in seed is influenced by environmental factors that lead to low O_2 levels and desiccation (Deaker, *et at.,* 2004), the possibility of a long term presence of Rhizobia inoculum in chipilin needs to be analyzed.

In conclusion, results suggest that *Rhizobium leguminosarum biovar* will provide the most nitrogen of the strains evaluated, but this needs to be confirmed by measuring the amount of nitrogen supplied to chipilin. The results of this study point to the importance of identifying the Rhizobia inoculating chipilin plants on the control treatment to specie to preclude the possibility of contamination.

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CHAPTER 5

CONCLUSIONS

Chipilin can be successfully grown in Massachusetts with an accumulated yield that can reach more than 7,200 kg·ha^{-1} with the application of 280 kg·ha^{-1} of nitrogen. However, based on this research the application of 80 kg·ha^{-1} appears to be sufficient for optimum yield; while nitrogen applied in addition to 80 kg·ha^{-1} does not significantly increase yield, it does enhance leaf color.

In the 2011 field evaluation, the decrease in yield with nitrogen applications above 120 kg·ha^{-1} can be due to the increase in soluble salt concentrations. It is speculated that this is due to the use of drip irrigation and plastic mulch. The plastic mulch will protect the soil from rainfall events and thus there will be less leaching compared to crop grown on bare soil. This reduction in yield in 2011, which did not occur in 2012, may be due to the fact that the average air temperature was significantly higher in 2011 which may have caused higher soil moisture evaporation compared to 2012, leading to higher salt concentration in 2011 compared to 2012.

Nodule found on the chipilin roots in the control treatments in the 2012 field experiment and the greenhouse experiment suggest that the source is either inoculum presence in the seed or contamination during the set up of the experiment; it could be also a combination of these two scenarios.

According to results obtained on accumulated yield, dry weight, plant length and number of nodules per plant differences among the Rhizobia strain evaluated, *Bradyrhizobium* USDA 3384 is not recommended for inoculations on chipilin. With an accumulated yield of 6,832.4 kg·ha^{-1} and the highest number of nodules per plant, *Rhizobium leguminosarum biovar* seems to be the most promising strain to provide nitrogen among the strains evaluated.

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APPENDIX A

Modified Arabinose Gluconate (MAG) bacteria medium growth.

Quantities are per liter of medium

		grams·L^1
HEPES		1.3
MES		1.1
Yeast Extract		1.0
L-arabinose		1.0
D-Gluconic Acid (sodium salt)		1.0
KH2PO4		0.22
NA2SO4		0.25
Stock solution (solution concentration)		
		ml
NH4Cl	16g/100ml	2.0
FeCl3	0.67g/100ml	1.0
CaCl2 (dehydrate)	1.5g/100ml	1.0
MgSO4 (heptahydrate)	18g/100ml	1.0

Adjust to pH 6.6w/KOH. Autoclave 20-30 minutes at 120C

* Add 18 g Bacto-Agar per liter for solid media

Source: USDA (www.usda.gov)

N-DURE label, *Rhizobium leguminosarum biovar phaseoli.*

INTX
MICROBIALS, LLC

N-DURE
A Peat-Based Inoculant for Dry Bean
Contains: Rhizobium leguminosarum biovar phaseolii

Product No: 6003
Net Weight: 4 lbs. 11 oz. (Inoculates 1,500 lbs., 25 bu., or 30 units of seed)
Package Configuration: 6 units per case, 48 cases per pallet

DIRECTIONS FOR APPLICATION:
- The optimum method for applying N-DURE is by using the slurry method.
- Dampen the seed with non-chlorinated, clean, cool water at a rate of 8.5 oz. of water per 50 pounds of seed.
- Add appropriate amount of inoculant (2.5 oz. /50 pounds of seed) and mix water, seed, and inoculant thoroughly until seed is uniformly coated.
- This method should be done in a container outside of the planter box.
- Allow 1-3 minutes for mixture to dry and then plant as soon as possible.
- N-DURE can also be applied dry directly onto the seed. Mix seed and inoculant thoroughly until seed is uniformly coated. Layering seed and inoculant will aide in this process.
- Applying the inoculant dry is also recommended for seed that is pre-treated with fungicide. However, maximum seed adhesion will not be obtained by applying this product dry.
- **FOR SOILS THAT HAVE NEVER BEEN HOST TO DRY EDIBLE BEANS, APPLY N-DURE AT A 1.5-2.0X RATE.**

STORAGE OF N-DURE:
- STORE IN A COOL, DRY PLACE OUT OF DIRECT SUNLIGHT
- STORE PRODUCT BETWEEN 40 AND 77º F
- OPEN PACKAGE ONLY WHEN READY TO USE
- USE BEFORE EXPIRATION DATE
- **N-DURE CARRIES A ONE-SEASON SHELF LIFE.**

N-DURE IS NOT A PLANT FOOD PRODUCT.

PRECAUTIONARY INFORMATION:
Although contents of this package are completely organic, avoid prolonged or repeated skin contact and inhalation. INTX Microbials, LLC recommends the use of a dust mask, safety glasses, and protective gloves when applying humus inoculants.

MINIMUM GUARANTEE:
This inoculant guarantees a minimum of 200 million (2×10^8) viable *Rhizobium leguminosarum biovar phaseoli* cells per gram.

LIMITED WARRANTY:
INTX MICROBIALS, LLC GUARANTEES THIS CULTURE TO PRODUCE SATISFACTORY NODULE FORMATION AND NITROGEN FIXATION UNDER FAVORABLE CONDITIONS OF SOIL AND CLIMATE WHEN USED BEFORE EXPIRATION DATE AND APPLIED UNDER THE MANUFACTURER'S SPECIFICATIONS, OR PURCHASE PRICE WILL BE REFUNDED. AN INTX MICROBIALS, LLC REPRESENTATIVE MUST BE NOTIFIED OF ANY FIELD COMPLAINT WITHIN FIFTY (50) DAYS AFTER PLANTING. MANY FACTORS OTHER THAN NODULATION AFFECT CROP PERFORMANCE. INTX MICROBIALS, LLC ASSUMES NO RESPONSIBILITY FOR LOSS OR PARTIAL LOSS OF CROP FROM ANY CAUSE WHATSOEVER. THIS LIMITED WARRANTY IS IN LIEU OF ALL OTHER WARRANTIES, EXPRESSED OR IMPLIED. THE LIMITED WARRANTY IS VOID WHERE PROHIBITED BY LAW. IF YOU HAVE ANY QUESTIONS ABOUT THIS PRODUCTS OR ANY OTHER INTX MICROBIALS, LLC PRODUCTS, CALL (219) 474-5510 FOR ASSISTANCE.

200 W. Seymour ǀ P.O. Box 62 ǀ Kentland, IN 47951
219-474-5510 ǀ 219-474-3700 fax ǀ 800-350-4789 ǀ www.intxmicrobials.com

APPENDIX C

N-DURE label, *Bradyrhizobium sp.* (Vigna).

INTX
MICROBIALS, LLC

N-DURE
A Peat-Based Inoculant for Peanuts and Lima Beans
Contains: Bradyrhizobium sp. (Vigna)

Product No: 2003
Net Weight: 8 ounces (Inoculates 100 lbs of seed)
Package Configuration: 54 packages per case, 48 cases per pallet

DIRECTIONS FOR APPLICATION:
- The optimum method for applying N-DURE is by using the slurry method.
- Dampen the seed with non-chlorinated, clean, cool water at a rate of 6.5 oz. of water per 50 pounds of seed.
- Add appropriate amount of inoculant (4 oz. /50 pounds of seed) and mix water, seed, and inoculant thoroughly until seed is uniformly coated.
- This method should be done in a container outside of the planter box.
- Allow 1-3 minutes for mixture to dry and then plant as soon as possible.
- N-DURE can also be applied dry directly onto the seed. Mix seed and inoculant thoroughly until seed is uniformly coated. Layering seed and inoculant will aide in this process.
- Applying the inoculant dry is also recommended for seed that is pre-treated with fungicide. However, maximum seed adhesion will not be obtained by applying this product dry.
- **FOR SOILS THAT HAVE NEVER BEEN HOST TO PEANUTS, APPLY N-DURE AT A 1.5-2.0X RATE OR USE N-DURE WITH A LIQUID INOCULANT PRODUCT FROM INTX MICROBIALS, LLC.**

STORAGE OF N-CHARGE:
- STORE IN A COOL, DRY PLACE OUT OF DIRECT SUNLIGHT
- STORE PRODUCT BETWEEN 40 AND 77º F
- OPEN PACKAGE ONLY WHEN READY TO USE
- USE BEFORE EXPIRATION DATE
- **N-DURE CARRIES A ONE-SEASON SHELF LIFE.**

N-DURE IS NOT A PLANT FOOD PRODUCT

PRECAUTIONARY INFORMATION:
Although contents of this package are completely organic, avoid prolonged or repeated skin contact and inhalation. INTX Microbials, LLC recommends the use of a dust mask, safety glasses, and protective gloves when applying humus inoculants.

MINIMUM GUARANTEE:
This inoculant guarantees a minimum of 200 million (2×10^8) viable *Bradyrhizobium sp. (Vigna)* cells per gram.

LIMITED WARRANTY:
INTX MICROBIALS, LLC GUARANTEES THIS CULTURE TO PRODUCE SATISFACTORY NODULE FORMATION AND NITROGEN FIXATION UNDER FAVORABLE CONDITIONS OF SOIL AND CLIMATE WHEN USED BEFORE EXPIRATION DATE AND APPLIED UNDER THE MANUFACTURER'S SPECIFICATIONS, OR PURCHASE PRICE WILL BE REFUNDED. AN INTX MICROBIALS, LLC REPRESENTATIVE MUST BE NOTIFIED OF ANY FIELD COMPLAINT WITHIN FIFTY (50) DAYS AFTER PLANTING. MANY FACTORS OTHER THAN NODULATION AFFECT CROP PERFORMANCE. INTX MICROBIALS, LLC ASSUMES NO RESPONSIBILITY FOR LOSS OR PARTIAL LOSS OF CROP FROM ANY CAUSE WHATSOEVER. THIS LIMITED WARRANTY IS IN LIEU OF ALL OTHER WARRANTIES, EXPRESSED OR IMPLIED. THE LIMITED WARRANTY IS VOID WHERE PROHIBITED BY LAW. IF YOU HAVE ANY QUESTIONS ABOUT THIS PRODUCTS OR ANY OTHER INTX MICROBIALS, LLC PRODUCTS, CALL (219) 474-5510 FOR ASSISTANCE.

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