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Investigating Limitations to Nitrogen Fixation by Leguminous Cover Crops in South Texas

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INVESTIGATING LIMITATIONS TO NITROGEN FIXATION BY LEGUMINOUS COVER CROPS IN SOUTH TEXAS

A Thesis

by

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Many farms use leguminous cover crops as a nutrient management strategy to reduce their need for nitrogen fertilizer. This practice depends on a symbiotic relationship between legumes and nitrogen-fixing rhizobia. Sometimes, despite inoculation with rhizobial strains, this symbiosis fails to form. Such failure was observed in a 14-acre winter cover crop trial in the Rio Grande Valley of Texas when three legume species produced no signs of nodulation and nitrogen fixation. This study examined nitrogen, phosphorus, moisture, micronutrients and native microbial communities as potential causes for the failure and assessed arbuscular mycorrhizal fungi as an intervention to improve nodulation outcomes. Results from two controlled studies confirm moisture and native microbial communities as major factors in the nodulation failure. Micronutrients showed mixed impacts on nodulation depending on plant stress conditions. Nitrogen and phosphorus deficiencies however were not likely causes, nor was mycorrhizal inoculation an effective intervention to improve nodulation results.

DEDICATION

I would like to dedicate this thesis to successful mutualisms – both ecological and interpersonal. The loving marriage of my parents, Mary and Jay Kasper, is my first and best example of a long-lasting, mutually-beneficial relationship. The legume-rhizobia symbiosis at the heart of this research is a variation on the same general theme. Cooperation creates vulnerability, but it is comforting that so many examples of healthy mutualisms exist, nonetheless.

ACKNOWLEDGMENTS

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CHAPTER I

INTRODUCTION

Cover Cropping in the Rio Grande Valley

Cover cropping is a conservation agriculture practice in which plants are grown as a strategic investment in soil health rather than for a harvestable yield (Sarrantonio and Gallandt 2003). Once a common agronomic practice to ensure long-term productivity in soils, cover crop usage faded during the Green Revolution as synthetic fertilizers and pesticides became dominant agricultural technologies in the United States (Groff 2015). However, interest in cover cropping has resurfaced in recent years and survey data suggest that the number of farms with cover crops and the number of acres covered have both increased (CTIC 2017). The benefits of cover cropping include increased soil organic matter, water infiltration and retention, soil fertility and nutrient retention, weed suppression, and pest life cycle disruption (Fageria et al. 2005; Snapp et al. 2005).

Congruent with national trends, farmers in the Rio Grande Valley, a major agricultural region in deep south Texas, show increased interest in cover crops. However, many questions remain regarding effective implementation. What species and combinations of cover crops are appropriate for this region, especially during the harsh summer season? When are the ideal times to plant and terminate for each cover crop species and within each cropping system? What termination methods work best, especially for organic growers limited to mechanical, rather than chemical, options?

Cover cropping has been a driver of soil health improvements in other regions in the U.S., but published research from these areas does not always apply to the semi-arid, subtropical Rio Grande Valley (Rugg 2016). Local farmers and researchers are working together to determine effective cover crops for this region through efforts like the Subtropical Soil Health Initiative (SSHI), a partnership between the National Center for Appropriate Technology (NCAT), the University of Texas Rio Grande Valley, Hilltop Gardens in Lyford, Texas, and PPC Farms in Mission, Texas. This project works with farmers on multi-acre trials seeking better answers to their questions about cover cropping in South Texas. In the past two years, this effort has helped mitigate some of the risk individual farmers face during the experimental phase of cover crop implementation and has raised new questions about cover crop usage in the Rio Grande Valley. One of those questions, and the focus of the research presented in this thesis, concerns effective nitrogen fixation by leguminous cover crops.

Leguminous Cover Crops: The Promise and Peril

Soil fertility benefits are a major draw for farmers considering cover crops. The prospect of paying less for nitrogen fertilizer is economically attractive, especially for organic growers who are restricted from using synthetic nitrogen and often rely on more expensive nitrogen sources (Klonsky 2012). In comparison to other benefits of cover cropping which often require several years of implementation to see significant impacts, reduced nitrogen fertilizer needs are often observed in the next season following a cover crop cycle (Cherr et al. 2006). Soil fertility improvements occur because legumes have a unique ability to partner with rhizobia, soil bacteria that can convert atmospheric nitrogen into plant-accessible forms (Dilworth and Glen 1984). The legume-rhizobia symbiosis is estimated to contribute 50-70 Tg of nitrogen each year to the global nitrogen budget, 18-26 Tg of which is within agricultural systems (Smil 1999; Herridge et

al. 2008). To replace current levels of legume derived N with synthetic fertilizer N would cost $7 to 10 billion each year (Graham and Vance 2003).

In addition to its economic value, the legume-rhizobia relationship avoids some of the negative externalities of synthetic fertilizer, a major source of agricultural greenhouse gas emissions and watershed degradation (Vitousek et al. 1997; Crews and Peoples 2004). However, biological nitrogen fixation is far from foolproof. It relies on a delicate partnership between two species and its success demands that niche requirements are met for both species (Denison and Kiers 2004; Graham 2007). Nodulation failure remains a common problem especially for introduced legumes whose rhizobial partners are not already present in the soil (Graham 1981; Miller and May 1991; Materon and Eubanks 2008). Legumes and rhizobia have different nutritional and environmental requirements, so the presence of seemingly healthy plants above ground is no guarantee that the bacteria below ground are also thriving and fixing nitrogen (Brockwell et al. 1991; O'Hara 2001).

In the first year of winter cover crop trials conducted by the SSHI, nodulation failure was a concerning surprise. Fourteen acres of leguminous cover crops – forage pea (*Pisum sativum*), hairy vetch (*Vicia villosa*), and crimson clover (*Trifolium incarnatum*) – were inoculated with peat-based rhizobial inoculants at planting. Nitrogen fixation was a priority for our farm partners in preparation for a spring sorghum crop. Aboveground, the plants appeared healthy, but root checks for nodulation during the season showed no signs of nodules for any of the legume species. The legumes still provided some benefits like weed suppression and increased organic matter but fell short on a primary goal of nitrogen fixation.

This nodulation failure launched a year-long effort of conversations with farmers and other experts, a literature review for published explanations, and several experiments to try to

better understand what factors may have been responsible for this result and what, if any, interventions might be possible. This thesis presents the results of this investigation. Chapter II includes the results from a greenhouse experiment that tested a few of the potential barriers to nodulation (moisture, micronutrients, phosphorus, nitrogen, and competitive soil microbes) to determine the most active players in the nodulation failure. Chapter III presents the results of a follow-up growth-chamber experiment on micronutrients and attempts to understand seemingly conflicting results with the original greenhouse experiment. Finally, Chapter IV provides an overview of the findings and lessons learned in this project, offers suggestions for farmers aiming to maximize nitrogen fixation on their own farms, and delineates several avenues for further research.

The Legume-Rhizobia Symbiosis

Legumes and their rhizobial partners are typically found together in their native habitats (Woomer et al. 1988). When legumes are introduced outside of their native ranges, some can recruit a replacement symbiont from the new environment (Martinez-Romero 2009). Most agricultural legumes, however, rely on the introduction of their original rhizobial partner through an inoculant (O'Hara et al. 2014). The earliest inoculation methods relied on the transfer of soil and its associated microbiology from an area where the legume was observed to form nodules (Fred et al. 1932). Since 1895, the most common method of inoculation has been peat-based, although liquid and granular options have also been developed (Brockwell and Bottomley 1995).

Whether rhizobia are native or introduced through an inoculant, the pathway to nitrogen fixation begins with the bacteria's infection of the plant root. The rhizobia recognize signal compounds from their host plant's roots and adhere to the root hairs. Once adhered, rhizobia begin synthesizing and exporting a nodulation factor to stimulate the nodule formation process in

their host (Gage 2004; Rodriguez-Navarro et al. 2007). This signals the plant to begin deforming its root hairs around the bacteria, initiating nodule formation (Brewin 1991; Oldroyd et al. 2011). The rhizobia grow and reproduce within the root hair curl and form an infection pocket (Murray 2011). This infection pocket develops into a branching infection thread, providing the rhizobia a pathway to invade the developing nodule cells (Gage 2004; Murray 2011). Within each nodule, the central pink or red infected tissue is responsible for nitrogen fixation. It is surrounded by inactive tan plant tissue called the inner cortex which contains the vascular tissue for the nodule. The entire structure is surrounded by endodermis and lenticel layers which control what enters and leaves the nodule (Walsh 1995). As illustrated in Figure 1.1, active nodules display a

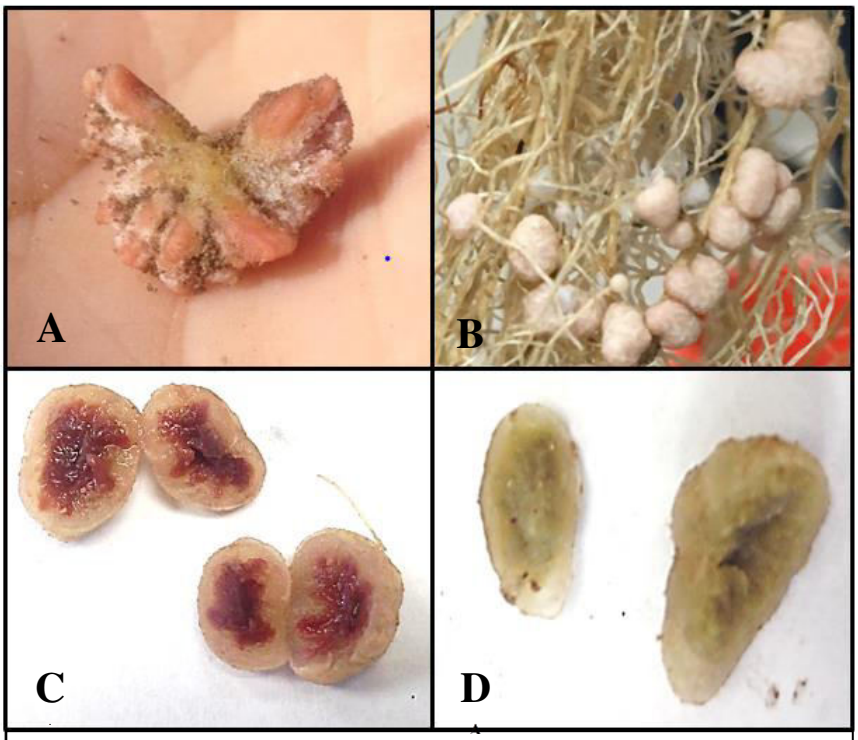


Figure 1.1 Example nodules – A shows an indeterminate nodule from sunn hemp (*Crotalaria juncea*) while B shows several round determinate nodules from cowpea (*Vigna unguiculata*). C shows two active cowpea nodules. Active nodules are pink, red, or red-brown inside, while inactive nodules are green, grey, or tan (D).

(Photo credit: S. Kasper)

different color in their infected tissue than inactive nodules. The distinct red-pink color of active nodules occurs due to the presence of leghemoglobin (O'Hara et al. 2014).

Leghemoglobin, like hemoglobin in vertebrate blood, is an oxygen-binding molecule that creates low free-oxygen concentrations within the infected nodule tissue (Dilworth and Glenn 1984). Low free-oxygen concentrations are important for the proper function of the nitrogenase, the key enzyme which converts dinitrogen into plant-accessible ammonia (Howard and Rees 1996). Rhizobia and the surrounding plant cells need oxygen for respiration, but free oxygen can irreparably damage the MoFe protein components of nitrogenase and repress further synthesis of replacement proteins (Appleby 1984). Leghemoglobin balances the needs of respiration and nitrogenase activity and provides a convenient visible indicator of nodule activity. Nodules with no color difference between the inside and outside of the nodule lack hemoglobin and likely never contributed to nitrogen fixation, while green or grey infected tissue indicates formerly active nodules that have senesced (O'Hara et al. 2014).

Legume nodules vary widely across species but can generally be categorized as indeterminate or determinate (Figure 1.1). Certain legumes including pea, vetch, clover, and sunn hemp have indeterminate nodules which grow outward in a fan pattern through cell division. Other legumes like soybean and cowpea develop determinate nodules which grow into round balls through cell expansion (Hirsch 1992).

Barriers to Effective Nitrogen Fixation

Barriers to nitrogen fixation can arise at any stage in nodule development. Ineffective inoculation, failed nodule initiation, inactive nodules, and occasional shedding of fully formed nodules have all been observed. Problems with nitrogen fixation typically fall into four categories: mineral nutrition, abiotic soil conditions, toxicity issues, and biotic conditions. Not all

these factors are relevant in the Rio Grande Valley or in the nodulation failure at Hilltop Gardens, but they provide context for the range of concerns a legume farmer might face.

Mineral Nutrition

Nitrogen Excess. Nodulation and nitrogen fixation can be inhibited by high field nitrogen levels (Graham 1981). High levels of nitrate in proximity to the nodules inhibits nitrogenase activity through a feedback mechanism, thereby reducing nitrogen fixation (Serraj et al. 1999). Above a certain concentration, excess nitrogen can inhibit nodule initiation entirely (Walley et al. 2005). Estimates for nitrogen levels that will eliminate nodulation vary widely. Some authors suggest a lower value of 50 kg/ha while others report nodulation for certain varieties at nitrogen levels as high as 120 kg/ha (Walley et al. 2005). Tolerance of nitrogen fixation to high soil nitrate levels varies across legume species and even among genotypes of the same species (Singh and Usha 2003).

Phosphorus Deficiency. Nitrogen fixation requires higher phosphorus levels than plant growth for nodule initiation, growth and activity (Israel 1987), and phosphorus deficiencies are commonly implicated in legume productivity issues (Graham 1981; O'Hara 2001). Nodules typically have phosphorus contents between 0.72 and 1.2 percent and are a strong P sink within the plant, especially under stressful environmental conditions (Zahran 1999). Phosphorus deprivation is associated with decreased nodule tissue formation and low rates of nitrogen fixation (Jakobsen 1985; Hogh-Jensen et al. 2002). Co-inoculation with mycorrhizae can sometimes improve nitrogen fixation, due to mycorrhizae's contributions to plant nutrition as a phosphorus scavenger (Chalk et al. 2006). In field tests of mycorrhizae, however, results of inoculation depended on the status of the native mycorrhizal population (Ortas 2003).

Micronutrient Deficiency. Legume-rhizobia symbiosis requires micronutrients including boron, cobalt, copper, iron, manganese, molybdenum, nickel, selenium, and zinc, sometimes at higher rates than the plant or free-living bacteria require alone (O'Hara 2001). Molybdenum's importance as a component of nitrogenase is well known, and it is often included on pre-season soil tests for legumes ((O'Hara 2001; Seefeldt et al. 2009).). Although less widely recognized than molybdenum, each of the other micronutrients is also critical for nodule formation or nitrogen fixation. Micronutrient deficiencies can be a serious impediment to the effective implementation of a leguminous cover crop (O'Hara et al. 1988; Gonzales-Guerrero et al. 2014). The role of each micronutrient in these processes will be discussed in greater depth in Chapter III.

Abiotic Factors

Temperature. Each legume species has an optimal soil temperature range for nodulation and nitrogen fixation that is not always identical to its optimal range for vegetative growth (Graham 1981). For example, guar, a highly heat- and drought-tolerant legume, can grow well at temperatures at and above 40^{o}C, but its nodule formation and nitrogen fixation rates are greatly reduced when temperatures exceed 37^{o}C (Arayangkoon et al. 1990). Temperature thresholds for other legumes are often much lower. Clover, for instance, shows limited nitrogen fixation when soil temperatures exceed 30^{o}C and common bean struggles similarly above 33^{o}C (Zahran 1999). Sometimes nodulation is inhibited entirely under high temperatures, and nodules that do form are often inactive. Nitrogen fixation potential may not recover until the legume returns to its optimal temperature zone for up to two weeks (Hungria and Franco 1993). Although not typically a concern in the Rio Grande Valley, low soil temperatures (13^{o}C or below) also inhibit or eliminate nodule initiation and nitrogenase activity (Bordeleau and Prevost 1994).

Water Stress. Observed as early as 1893, the importance of adequate moisture for nodule formation and activity has long been acknowledged (Fred et al. 1932). Legumes require more moisture for nitrogen fixation than for plant growth (Kirda 1989; Hunt et al. 1981).. Since nodules receive water through volumetric phloem flow, even small changes in leaf water potential can lead to major reductions in nodule water supply (Walsh 1995). Water is required to export nitrogen products from the nodules to the rest of the plant, so when their water supply is diminished, nitrogen products build up in the nodule and inhibit further fixation (Serraj 1999).

Extreme moisture stress can inhibit nodule initiation or cause nodule shedding in certain legume species (Kirda 1989; Williams and De Mallorca 1984). Mycorrhizal inoculation may also help alleviate drought induced oxidative damage and preserve nodule function under moisture stress conditions (Ruiz-Lozano et al. 2001). Excess moisture can also reduce nitrogen fixation potential if insufficient oxygen for rhizobial respiration is available (Brockwell et al. 1995).

pH. Legume tolerance to acidity and alkalinity varies across species, but most do best in a neutral to slightly acidic environment with frequent nodulation failures in highly acidic soils (Brockwell et al. 1995; Zahran 1999). There is a critical threshold near 5.0 pH, below which nitrogenase activity is delayed and significantly reduced compared to neutral and more moderately acidic soils (Schubert et al. 1990).

Acidic conditions interfere with the rhizobia's ability to attach to the plant's root hair and launch the infection process (Zahran 1999). pH is also a major determinant of soil nutrient availability and most essential plant nutrients decline in plant accessibility in extremely acidic soils (Binkley and Vitousek 1989). Molybdenum, which is an especially important component of nitrogenase, is strongly adsorbed to soil oxides when pH is below 5.5, making it far less soluble than at higher pH levels (Kaiser et al. 2005).

The effect of soil acidity can be buffered by the inoculant method used. In an experiment testing this interaction, liquid inoculant was observed to fail at a pH of 5.4, and only granular was effective at pH 4.4 (Rice et al. 2000). Alkaline soil itself is not typically a problem for rhizobia and experimental legumes have grown well up to a pH of 10. However, high pH soil is often associated with high salinity and reduced nutrient availability, both major obstacles to rhizobia survival and nitrogen fixation success (Bordeleau and Prevost 1994).

Salinity. Although rhizobia usually survive at extremely high levels of salt, many legumes have low resistance to salinity, limiting N fixation in saline soils (Bordelau and Prevost 1994). High salt concentrations reduce nodulation by impeding the root hair colonization process required for rhizobial invasion (Zahran 1991). If nodules form, reduced nodule respiration rates under saline conditions may limit N fixation rates (Zahran 1999).

Major differences in salt tolerance exist across legume species and among different cultivars of the same species. Alfalfa, yellow-lupin, faba bean, and mesquite are particularly salt-tolerant and could be options for farmers facing saline conditions (Zahran 1991). Variety trials in chickpea have detected genotypes that can successfully form nodules and fix nitrogen at an electroconductivity of 6.2 ds m^{-1}, double the salinity levels that impede more salt-vulnerable chickpeas (Rao et al. 2002). Mycorrhizal inoculation may also protect plants from salt stress, potentially through more efficient nutrient access which can improve plant resilience to environmental stress (Azcon and El-Atrash 1997).

Toxicity

Nodulation failure can occur if rhizobia are killed through exposure to toxic substances before they are able to colonize the plant root. Known toxins to rhizobia include fungicides, solvents, alcohols, and disinfectants (O'Hara et al. 2014). Heavy metals including lead,

cadmium, nickel, chromium, copper, and zinc are also toxic to both rhizobia and legumes in high concentrations and cause declines in nodulation, nitrogen fixation, and plant growth (Wani et al. 2007). Rhizobial inoculants should not be exposed to any of these substances before or during inoculation or planting. This can be particularly tricky for fungicides which are often applied as seed coat treatments. However, where legumes face serious fungal threats and fungicidal seed coatings are desired, granular inoculants may help protect rhizobia from direct contact with fungicides and improve nodulation success (Graham 1981).

Some legume seed coats produce exudates that are toxic to rhizobia. The toxicity and concentration of seed coat exudates vary across species leading to low success rates of seed pre-inoculation for those with higher concentrations and more toxic exudates (Deaker et al. 2004). Antibiotic seed exudates are a helpful adaptation for plants to protect young, vulnerable seedlings from immediate bacterial infection. Unfortunately, these exudates cannot distinguish between potential pathogens and potential symbionts and kill both indiscriminately (Bowen 1961). These antibiotic seed coat effects can be mitigated by waiting to inoculant until just before seeding (O'Hara et al. 2014).

Biotic Factors

Competitive native microorganisms. Local rhizobia are widespread in the Rio Grande Valley in association with native or naturalized legumes like burr medic, and introduced legumes like common bean, cowpea, and sunn hemp (Eubanks 2005). Better adapted native or naturalized rhizobia have been observed to outcompete introduced strains when competing for root infection sites (Graham 1981). Unfortunately, superior infectivity (ability to colonize roots) is not always accompanied by increased effectivity (ability to fix nitrogen) in rhizobia (O'Hara et al. 2014).

Poor inoculation adhesion and survival. Legumes show the best nodulation results when certain thresholds of rhizobial inoculant per seed are achieved. The threshold levels are 100,000 rhizobia/seed for large-seeded legumes, 10,000/seed for medium-seeded, and 1000/seed for small-seeded (O'Hara et al. 2014). Inoculation method affects whether these thresholds are achieved (Deaker et al. 2004). Often, peat-based inoculant is mixed into lightly moistened seed, then applied via seed spreader or drill. Inconsistent inoculant coverage of seed can be compounded by inoculant loss during the seeding process. Moisture evaporation during seeding combined with the vibrations of the tractor or other seeding device can dislodge the inoculant (Elegba and Rennie 1984). Adhesive additives like gum arabic, methyl cellulose, or vegetable oil can improve rhizobial adherence and increase the possibility of successful infection and nodulation in plant roots (Elegba and Rennie 1984; Hoben et al. 1991).

One final concern is initial inoculant quality. In many countries, including the United States, inoculant quality control is left to the discretion of the manufacturer (Deaker et al. 2004). A farmer cannot assess quality just by looking at the inoculant. When lab tested, inoculant products regularly show lower levels of rhizobia than those recommended by researchers or claimed by the manufacturer (Lupwayi et al. 2000). Even if inoculant quality is high at the point of manufacture, it can also lose viability between production and use if exposed to high or low temperatures or kept past the expiration date (O'Hara et al. 2014). Rhizobial inoculants are a living product, unlike many other farm inputs and amendments, and must be stored under conditions that maximize rhizobial survival.

CHAPTER II

IMPACT OF MOISTURE, MICRONUTRIENTS, PHOSPHORUS, NITROGEN, AND SOIL STERILIZATION ON NODULATION

Abstract

Leguminous cover crops are a nutrient management strategy that can reduce a farm's need for nitrogen fertilizer. Biological nitrogen fixation depends on a symbiotic relationship between legumes and nitrogen-fixing rhizobia. Under certain conditions, despite inoculation with appropriate rhizobial strains, this symbiosis fails to form. Such failure was observed in a 14-acre winter cover crop trial in the Rio Grande Valley of Texas when three legume species produced no signs of nodulation and nitrogen fixation. This study examined nitrogen, phosphorus, moisture, micronutrients and biotic interference as potential causes for the failure and assessed arbuscular mycorrhizal fungi as a potential intervention to improve nodulation outcomes. Results from a controlled greenhouse study confirm moisture and micronutrient deficiencies as major suspects in the nodulation failure. Higher soil moisture and micronutrient levels both significantly increased nodule biomass. Nitrogen and phosphorus deficiencies however were not likely causes, nor was mycorrhizal inoculation an effective intervention to improve nodulation results. A better understanding of the limitations of nitrogen fixation could help support farmers in the Rio Grande Valley and elsewhere in their efforts to convert to more sustainable nutrient management practices.

Introduction

Farmers have long grown legumes as centerpieces of their agricultural systems, from lentils in Mesopotamia to beans in early Mesoamerica, and soy in ancient China (Zohary and Hopf 1973; Hymowitz and Newell 1981; Sweeney and McCouch 2007). Widespread adoption of legumes is due in part to their unique ability to partner with rhizobia, soil bacteria that can convert atmospheric nitrogen into plant accessible forms. Historically, the legume-rhizobia symbiosis was a primary mechanism to maintain adequate nitrogen levels in agricultural soils over years of cultivation (Gliessman 2015).

A shift away from leguminous nitrogen sources to synthetically produced fertilizer sparked the massive yield increases of the Green Revolution (Smil 2002). This shift has also been associated with significant environmental costs. Surplus nitrogen enters waterways through runoff, leading to nitrate pollution, algal blooms, and hypoxic environments that damage marine ecosystems (Vitousek et al. 1997; Crews and Peoples 2004). Synthetic nitrogen production and application can also increase greenhouse gases emissions including nitrous oxide and carbon dioxide (McAllister et. al. 2012). In this context, biological nitrogen fixation with legumes is being revisited as a potential low-cost component of more sustainable nutrient management systems with reduced nitrogen runoff compared to conventional systems (Drinkwater et al. 1998).

Cover cropping is a conservation agriculture practice where plants are grown for their benefits to soil health and nutrition rather than their harvestable yield (Sarrantonio and Gallandt 2003). Farmers incorporating cover crops often include legumes for their contributions to sustainable nitrogen management. Although much research effort has already been directed towards maximizing efficiency and yield for major leguminous commodities like soy

(Salvagiotti et al. 2008), less attention has been paid to efficient nitrogen fixation by leguminous cover crops. Interest in cover cropping has burgeoned in recent years and survey data suggest that the number of U.S. farmers incorporating the practice and number of acres covered are both increasing over time (CTIC 2017). Cover cropping can help increase soil organic matter, improve water infiltration and storage and enrich soil ecosystems among other contributions to soil health (Snapp et al. 2005). Leguminous cover crops can be a cost-effective component of soil fertility management, especially for organic growers who often rely on more expensive nitrogen sources (Pimentel et al. 2005; Klonsky 2012).

Despite advances in microbiological science, agricultural technology, and inoculant production, nodulation failure in introduced legumes remains a common problem (Graham 1981; Miller and May 1991; Materon and Eubanks 2008). For example, in a large-scale field trial of leguminous cover crops in subtropical south Texas – 3 different leguminous species were inoculated with appropriate rhizobia and planted as cover crops during the winter season. They were intended to contribute positively to the nitrogen supply for the planned spring grain crop. Aboveground, the plants appeared healthy, but root checks for nodulation showed no signs of nodules for any of the legume species (Racelis et. al. 2019, unpublished data).

Large-scale failure of leguminous crops to contribute nitrogen can discourage widespread adoption. Further investigation is critical to better understand and avoid such failures and improve the efficiency of leguminous cover crops. Additional research could help improve nitrogen fixation efficiency for cover crop legumes, especially in regions where cover crop systems are in the early phases of adoption and implementation (Miller and May 1991).

Biological nitrogen fixation relies on a delicate partnership between plant and bacterial species and is maximized when niche requirements are met for both species (Denison and Kiers

2004; Graham 2007). The legume is the dominant partner in this mutualism and factors that limit plant health and photosynthetic capacity will likewise limit nitrogen fixation potential (Brockwell et al. 1995; Denison and Kiers 2004). Even when optimal legume conditions are met, rhizobial establishment can be inhibited by several other factors (Table 2.1).

This study examines the relative strength of various abiotic factors including moisture (Kirda 1989; Walsh 1995), micronutrient availability (O'Hara 2001), and phosphorus and nitrogen availability (Israel 1987; Walley et al. 2005), as well as biotic factors such as competition from native microbes (Eubanks 2005; O'Hara et al. 2014) to predict successful biological nitrogen fixation.

Ecological facilitation with other microorganisms such as arbuscular mycorrhizal fungi (AMF) can help improve plant health under environmental stress (George et al. 1995; Lynch 2019). Therefore, we also explore the potential of co-inoculation with arbuscular mycorrhizal fungi to buffer the legume-*Rhizobium* symbiosis from stressors and enhance nodulation and N fixation. The goal of this work is to improve the efficiency of leguminous cover crops as an appropriate and effective biological tool for the maintenance of nitrogen in subtropical soils.

Methods

Five separate factorial experiments were conducted concurrently in controlled greenhouse conditions (Edinburg, TX) to examine the association of abiotic (moisture, micronutrients, phosphorus, nitrogen) and biotic (presence of other soil micro-organisms) conditions on nodulation in a common cover crop legume, cowpea (*Vigna unguiculata*). In each experiment, the potential interaction of arbuscular mycorrhizal fungi as a predictor for the successful nodulation in cowpea was also examined.

For each experiment, Iron and Clay cowpea seeds (*Vigna unguiculata;* Johnny's Seeds, Winslow, ME) were soaked for 10 minutes in 55^{o} C water, then pregerminated for 3 days in petri dishes in the greenhouse at 30^{o}C. Pregerminated seeds were then transplanted into 15 cm plastic pots with 1500 g of a 1:1 mixture of perlite and soil obtained from the field where nodulation failure occurred (Hilltop Gardens, Lyford, TX). Field conditions for this soil are shown in Table 2.2. Nutrient extractions were conducted by Texas Plant and Soil Lab (Edinburg, TX). Nitrogen and phosphorus values are the average of 25 samples analyzed by Mehlich III extraction. Micronutrient values are from a single soil sample using the hot water method for boron extraction and DTPA for cobalt, copper, manganese, molybdenum, and zinc. Soil pH was measured using a multimedia pH meter (Bluelab, Tauranga, New Zealand) and soil texture was determined using the USDA NRCS Web Soil Survey and confirmed by hydrometer.

At transplant, a 1 ml solution of rhizobium inoculant solution (2 g inoculant/500 mL water; Verdesian Guard-N®, Cary, NC) was applied to the seed radicle. A mycorrhizal inoculant solution (1 g inoculant/500 mL water; Wildroot Organic, Austin, TX) was also applied at transplant to the cowpeas assigned to factorial treatments that included mycorrhizae (M+). In all experiments, cowpeas were grown for 75 days in greenhouse conditions. Daily temperature ranged between 28 ^{o}C and 36 ^{o}C on average, and relative humidity ranged between 52 and 86%. Soil pH measurements were taken initially upon planting (mean - 8.0 +/- 0.1) and monthly during the experiment to check for pH changes from nutrient solutions, but none were detected.

Pots were watered based on daily moisture measurements using a moisture meter (ProCheck 5TE, Pullman WA). Except where otherwise indicated below, the pots were watered with 150 mL of tap water (or the designated nutrient solution) whenever their soil moisture fell below a lower threshold of 5%. This amount of water raised the soil moisture to an upper target

of 15%. Tap water was used instead of deionized water in order to better simulate field conditions since both rainwater and local irrigation water sources carry trace minerals (Lindberg 1982; City of Edinburg 2018). However, in the absence of specific water testing for our nutrients of interest, exact treatment impacts cannot be determined. Levels listed in Table 2.2 should be considered lower thresholds. These lower thresholds (µg element/g dry soil) were calculated using the concentration for each nutrient solution, the total volume of solution applied, and the dry mass of the soil.

Moisture

The role of moisture on legume nodulation was examined under three moisture regimes in a 3 X 2 factorial design, with both moisture and mycorrhizae as main factors. Moisture levels were designated as *high* (soil moisture maintained between 15-25%), *mid-range* (soil moisture between 5-15%) or flood/drought *cycle* (ranging between near wilting point and field capacity soil moisture). In the high moisture treatment, pots received 200 mL of water when they reached a threshold of 15% soil moisture which raised them to field saturation (around 25%). For the flood/drought cycle, plants received 500 mL after 3 days below a threshold of 2.5% soil moisture. They were watered in two 250 mL increments to minimize leaching and runoff. The three-day wait was set based on the average time cowpeas took to wilt after reaching 2.5% soil moisture in a pre-trial assessment. Using a 3x2 factorial design, this experiment compared all combinations of three levels of moisture – high, mid, and cycle – and two levels of mycorrhizal inoculation – with (M+) or without (M-).

Micronutrients

We compared impact of addition of these micronutrients (copper (0.5 µM $CuSO_4$), cobalt (1.7 µM $CoCl_2$) boron (25 µMH_3BO_3), molybdenum (0.5 µM Na_2MoO_4), manganese (2 µM

$MnCl_2$), and zinc (2 µM $ZnSO_4$)) on root nodulation to a control with field level micronutrients (Table 2.2). Micronutrient concentrations were based on a modified Hoagland's solution (Taiz et al. 2015). Using a 2x2 factorial design, this experiment compared two levels of micronutrients – micronutrients added (Mi+) and field level (Mi-) – and two levels of mycorrhizal inoculation – M+ or M-.

Phosphorus

Different levels of phosphorus were tested including *field level* phosphorus (Table 2.2), *low* phosphorus (0.1 mM KH_2PO_4, 1.9 mM KCl), and *high* phosphorus (2 mM KH_2PO_4,).). Since phosphorus (target nutrient) was supplied as KH_2PO_4, potassium levels (non-target) were also raised. To avoid confounding the impacts of P and K on nodulation, low and field level P treatments were supplemented with potassium chloride (KCl) to match the K levels applied to the high P treatment (Table 2.2). Using a 3x2 factorial design, this experiment compared three levels of phosphorus–field, low and high – and two levels of mycorrhizal inoculation – M+ or M-.

Nitrogen

We compared the impact on nodulation of field level nitrogen (Table 2.2) to nitrogen levels both higher (5 mM CaN_2O_6) and lower (1/2 field level). Higher N treatments had calcium nitrate added in solution while lower N was achieved through a 50/50 mix of field soil and sand. Since the high nitrogen treatment also received 5 mM Ca (non-target) in addition to 10 mM N (target), low and field level N treatments were supplemented with calcium chloride (5 mM $CaCl_2$) to match the calcium levels applied to the high N treatment (Table 2.2). These adjustments were made to avoid confounding the impacts of Ca and N on nodulation. Using a

3x2 factorial design, this experiment compared three levels of nitrogen – low, field, and high – and two levels of mycorrhizal inoculation – M+ or M-.

Soil Sterilization

To isolate some of the potential effects of any active soil microbes impacting nodulation and nitrogen fixation, a sterilization treatment was added in which soil media was steam sterilized in an autoclave at 121 ^{o}C for 30 minutes before planting. Using a 2 X 2 X 2 factorial design, this experiment compared all combinations of following three factors – sterilized (S+) or unsterilized (S-) soil, with (M+) or without (M-) mycorrhizal inoculation, with (R+) or without (R-) rhizobial inoculation. Steam sterilization can affect soil pH as well as nutrient content and availability. Therefore, separate tests were conducted to determine the baseline pH, organic matter, and nutrient levels for the sterilized soil.

Data collection

During the final week before termination (days 68-74), maximum photosynthesis measurements (Asat) were taken for three replicates from each treatment using a LiCor 6400XT Portable Photosynthesis System. Asat was recorded at 2000 PAR after the assimilation value had plateaued and the stomatal conductance value exceeded a threshold of 0.05 mol m^{-2} s^{-1}. After 75 days, five replicates from each treatment were randomly chosen. Roots were cleaned and examined for nodules which were counted, weighed, and checked for internal color as an indicator of nitrogen fixation activity. Pink, red or brown nodules were counted as active while green, grey, tan, and any other color were considered inactive (O'Hara et al. 2014). Plants were then dried for at least 72 hours at 70^{o}C and the dry biomass of roots, stems, and leaves for each plant were recorded.

Data analysis

For the moisture, micronutrient, phosphorus, and nitrogen experiments, 2-way analyses of variance were conducted to compare the main effects of each factor and mycorrhizal inoculation and the interaction effect between that factor and mycorrhizae on nodule number, biomass, and activity and plant indicators including Asat, root, stem, leaf and total biomass, root to shoot ratio and nodule to plant ratio.. Multiple comparisons were performed using the Holm-Sidak method. When assumptions of normality and equal variance were violated, a Kruskal-Wallis 1-way ANOVA on ranks was employed, followed by Dunn's method for multiple comparisons. For the sterilization experiment, three-way ANOVAs were conducted to compare the main effects of sterilization, mycorrhizal inoculation, and rhizobial inoculation and the interaction effects among the three (SYSTAT™, San Jose, CA).

Results

Moisture

No significant interactions or effects of mycorrhizae were detected at the 0.05 level of significance for any of the plant or nodule indicators. No significant effects of moisture level were detected for the following variables: nodule number, nodule activity, max photosynthesis, leaf area, stem biomass, and root to shoot ratio (Table 2.3). Significant effects of moisture level were found for nodule biomass ($F(2,24) = 4.941$, $p = 0.016$), nodule to plant ratio ($F(2,24) = 3.713$, $p = 0.039$), root biomass($F(2,24) = 6.475$, $p = 0.006$), leaf biomass ($F(2,24) = 4.03$, $p = 0.031$), and total biomass ($F(2,24) = 3.456$, $p = 0.048$) (Figure 2.1).

Holm-Sidak multiple comparisons showed a significant difference only between high moisture and cycle treatments for nodule biomass ($p=0.013$), nodule to plant ratio ($p = 0.037$), leaf biomass ($p = 0.031$), and total biomass (0.046). Multiple comparisons showed significant

differences between both high/cycle and high/mid pairings for root biomass ($p = 0.008$; $p = 0.019$). No significant differences between mid and cycle moisture were detected.

Micronutrient

No significant interactions or effects of mycorrhizae were detected at the 0.05 level of significance for any of the plant or nodule indicators. No significant effects of micronutrient level were detected for the following variables: nodule number, nodule activity, max photosynthesis, leaf area, root to shoot ratio, stem, leaf, and total biomass (Table 2.3). Significant effects of micronutrient level were found for nodule biomass ($F(1,16) = 7.671$, $p = 0.013$), root mass ($F(1,16) = 4.515$, $p = 0.049$), and nodule to plant ratio ($F(1,16) = 10.179$, $p = 0.005$).

Phosphorus

Only the effect of phosphorus level on nodule percent activity was statistically significant at the 0.05 significance level (Table 2.4). The main effect for phosphorus level on nodule activity yielded an F ratio of ($F(2,24) = 5.467$, $p = 0.011$) indicating a significant difference among high P (M= 60, SD=14), low P (M= 33, SD=20) and field P (M= 46, SD= 18). Holm-Sidak multiple comparisons showed a significant difference only between high phosphorus and low phosphorus treatments ($p=0.009$). Differences among treatments for all other plant and nodule indicators were not significant.

Nitrogen

No significant interactions between nitrogen level and mycorrhizae were detected. For mycorrhizae, the only significant effect was for root biomass ($F(2,24) = 4.717$, $p = 0.04$). No other significant impacts of mycorrhizae were detected nor for nitrogen level for max photosynthesis, root biomass, or stem biomass (Table 2.4). Significant effects of nitrogen level were detected for nodule number ($F(2,24) = 8.163$, $p = 0.002$), nodule activity ($F(2,24) = 14.064$,

p = <0.001), nodule to plant ratio ($F(2,24) = 62.239$, p = <0.001), leaf area ($F(2,24) = 5.286$, p = 0.012), leaf mass ($F(2,24) = 5.522$, p = 0.01), total mass ($F(2,24) = 3.746$, p = 0.038), and root to shoot ratio ($F(2,24) = 10.34$, p = <0.001).

Multiple comparisons indicated that high nitrogen values significantly differed from below field values for leaf area (p=0.01), leaf biomass (p=0.008), and total biomass (p=0.034). High nitrogen differed significantly from both below field and field level N for nodule number (p=0.005; p=0.004), nodule activity (p<0.001; p=0.003), and root to shoot ratio (p<0.001, P=0.003). For nodule to plant ratio, all three nitrogen levels significantly differed (p<0.001, p<0.001, p=0.02).

High nitrogen treatment inhibited nodulation in all but one replicate. Due to the large number of zeros, nodule biomass data failed normality and equal variance. A Kruskal-Wallis one-way ANOVA on ranks was used instead and found significant differences in nodule biomass among the three nitrogen levels ($H(2) = 21.425$, $p < 0.001$). Dunn's method for pairwise comparisons among the nitrogen levels found that nodule biomass for the high nitrogen treatments were significantly less than field nitrogen ($Q = 4.053$, $p < 0.05$), and low nitrogen ($Q = 3.751$, $p < 0.05$), but that field nitrogen and low nitrogen did not vary significantly from each other ($Q = 0.295$, $p > 0.05$).

Sterilization

Significant interactions were detected between sterilization and rhizobia for both nodule number and nodule to plant ratio. In the case of nodule number, there were no significant differences between S+ and S- treatments overall, but there were significant differences between S+ and S- within R+ (p = 0.03) and R- (p = 0.032). The results for nodule to plant ratio showed a similar pattern with significant differences between S+ and S- within R+ (p <0.001) and R-

($p<0.001$). In this case, the nodule to plant ratio was differed significantly by sterilization ($F(7,32)=65.981$, $p < 0.001$). No other significant interactions among sterilization, mycorrhizae, and rhizobia occurred (Table 2.5).

The only significant effect of mycorrhizae was detected for nodule number ($F(7,32)=4.718$, $p=0.037$). Nodule number was also the only indicator to show a significant effect of rhizobia ($F(7,32)=7.533$, $p=0.01$). No other effects of mycorrhizae or rhizobia were detected.

In addition to effects already discussed on nodule number and nodule to plant biomass, significant effects of sterilization were detected for all other nodule and plant indicators including: maximum photosynthesis ($F(7,32)=11.907$, $p < 0.001$), leaf area ($F(7,32)=76.407$, $p<0.001$), nodule activity ($F(7,32)=50.645$, $p<0.001$), root biomass ($F(7,32)=6.872$, $p=0.013$), stem biomass ($F(7,32)=22.151$, $p<0.001$), leaf biomass ($F(7,32)=31.946$, $p<0.001$), total biomass ($F(7,32)=31.946$, $p<0.001$), and root to shoot ratio ($F(7,32)=24.533$, $p < 0.001$) (Table 2.3).

Nodule Biomass as a Predictor of Plant Biomass

Figure 2.2 shows the positive correlation between dry nodule biomass (g) and dry total plant biomass (g). Although the trend is strong ($r=0.771$, $p<0.001$), notable outliers are pictured in green. These outliers are the high nitrogen treatment which did not produce nodules and whose plant biomass was independent of nodule biomass. Additional correlation values for each of the treatment subsets shown in Table 2.6.

Discussion

Effective biological nitrogen fixation depends on the success of the legume-rhizobia symbiosis. The mutualism between these two species is ecologically complex, and effective management of this relationship requires a better understanding of the predictive factors for the

purpose of soil improvement. Although nodule biomass is an imperfect estimate of nitrogen fixation because the mere presence of nitrogenase does not indicate its activity level (Boote et al. 2008), nodule biomass can serve as a proxy for nitrogen fixation potential, since generally, more nodule tissue means more rhizobia, higher nitrogenase levels, and increased potential for nitrogen fixation (Voisin et al. 2003; Liu et al. 2011).

Other characteristics (such as nodule count) may not be as reliable. In our results, nodule count (abundance) hides some important information about the size of those nodules and is not the most useful indicator of different outcomes in nodulation and plant health. Significant differences in internal nodule color, an indicator of nodule activity, did not always align with differences in nodule biomass. For example, there were no differences in nodule activity across the moisture or micronutrient treatments which differed significantly in plant and nodule biomass, but there were significant differences across phosphorus treatments which showed no other differences for any of the other measured variables. Although not as simple as a nodule count, nodule biomass and activity checks are more accurate low-tech indicators for farm managers who employ leguminous cover crops for nitrogen management.

This research suggests that certain abiotic factors such as micronutrients and moisture were major determinants of nodule biomass in this agroecosystem. In contrast, phosphorus inaccessibility and excess nitrogen were not likely responsible for nodulation failure, nor was addition of mycorrhizae an effective intervention to improve nodulation results (Figure 2.1). When a legume enters a period of water stress, nodules are the first to lose their water supply since the process of nitrogen fixation is more sensitive to drought than plant growth (Serraj et al. 1999). In this experiment, periodicity of moisture seems more influential on nodulation than the total amount of moisture received over the entire season. For example, plants under the

flood/drought cycle regime received approximately the same amount of total water over the course of the experiment as mid-range moisture plants (3515 mL and 3445 mL respectively), but we found that acute drought/flood stress, which is similar to conditions in a dry-land agriculture or a flood irrigated setting, put the plants at a serious disadvantage compared to plants with metered moisture.

In a dryland farming situation, not much can be done to improve legume outcomes. One proposed option is to choose legumes based on drought tolerance. However, these results suggest that even cowpea, one of the most heat and drought tolerant legume options for South Texas, is vulnerable to the impact of moisture stress on nodulation and nitrogen fixation. Other studies on cowpea have shown that nodule water potential and nitrogenase activity show major declines after periods of droughts, even before leaf water potential shows any changes (Pararajasingham and Knievel 1990). Even minor 15% declines in photosynthesis in a moisture stress situation can be accompanied by 90% drops in nitrogenase activity (Venkateswarlu et al. 1989).

Our results suggest that in-field micronutrient deficiencies can limit nodulation. The addition of the micronutrient solution containing molybdenum, manganese, zinc, boron, copper, and cobalt increased nodule biomass 79% over the control (field level micronutrient). Fortunately for farm managers, conventional and organic micronutrient amendment options are available and could be applied to maximize nodulation of leguminous cover crops contingent on the results of a soil tests. Further research is required to pinpoint specific micronutrients that can facilitate improved nodulation in alkaline subtropical soils.

Nodulation in legumes has been known to regulated by rates of native nitrates (Herridge et al. 1984; Imsande 1986), and our results are congruent with these findings. Differences in nodule biomass between the nitrogen treatments in this experiment were highly significant, and

strongly predicted nodulation in our experiment. We found little or no nodulation in plants where excessive rates of N were added, reinforcing the recommendation that leguminous cover crops are best employed where nitrogen may be at a relative deficiency. In this specific agroecosystem, nodules did form at field level nitrogen (37.6 lbs/acre) and there was no significant difference between nodulation at field level N and below field level N. In farms where synthetic nitrogen inputs are unlikely to reach excessively high N levels, leguminous cover crops may assist in maximizing plant-available nitrogen while providing other benefits to soil health, such as weed control (Rugg 2016), and soil microbial biodiversity (Soti et al. 2016; McDaniel et al. 2014).

Microbial diversity plays a key function in soils. We found that plants in sterilized soil (with no major changes in soil nutrient levels) had significantly reduced nodule biomass and were less healthy by every measure than plants in native (unsterilized). In this case, the complex microbial communities associated with healthy soils were not only found facilitate the relationship between rhizobia and plant but are associated with various metrics of plant growth and vigor. A better understanding of the ecology of microbial communities (and the impact of introductions such as rhizobial and mycorrhizal inoculants) into agroecosystems is required to maximize the functionality of cover crops and overall soil health.

Mycorrhizal inoculation was not observed to significantly impact nodule biomass in any component of this trial. This result was a little counterintuitive considering the existing evidence for the benefits of tripartite symbiosis among legumes, rhizobia, and mycorrhizae (Ortas 2003; Koide 1991). This may be an artifact of the inoculant, which may have either lacked viable infective propagules or been poorly suited for soil and climate conditions. Based on these results, finding site-suitable mycorrhizal inoculants is highly recommended for farmers and farm

managers looking to employ this strategy on-farm. Another possibility is the general difficulty of observing the impact of mycorrhiza in a greenhouse setting in relatively small pots. The benefit from mycorrhizae comes from the ability of the hyphal network to expand its nutrient mining reach beyond the range that the plant's root system can access on its own. In a small pot setting where the root system can mine the available soil volume effectively, the potential benefit of mycorrhizae is lessened (Poorter et al. 2012).

One unplanned observation from this study has suggested that perhaps inoculation method (for both rhizobia and mycorrhiza) can play a strong role in establishment (Deaker et al. 2004; O'Hara et al. 2014). In this study, we expected that nodule initiation would likely fail for many of the treatments, given the common report of nodulation failure in the field. However, the only treatment in which nodulation was completely inhibited was the highest nitrogen treatment, for reasons discussed above. Commonly for field scale inoculation, a peat-based inoculant is applied to lightly wetted seeds, then applied via a seed spreader or drill. In our controlled study, a rhizobial inoculant slurry was pipetted directly onto pregerminated seed root radicles. We found that 92% of the cowpeas our experiment formed at least one nodule, while in a 2018 field trial only 38% of the cowpeas had the same result.

Obviously, an inoculation method as precise as the greenhouse method would not be feasible at a farm scale. That said, any alternative method for farm-scale inoculation that increases rhizobial adherence to seeds such as adhesive additives (gum arabic, methyl cellulose, or vegetable oil) would improve the possibility of successful infection and nodulation in plant roots and thus nutrient management (Elegba and Rennie 1984; Hoben et al. 1991). Although outside the scope of this investigation, agronomic practices are as critical as biological and

ecological factors in the task of maximizing nitrogen fixation efficiency by cover crops on South Texas farms.

Tables and Figures

Table 2.1 – Barriers to Rhizobial Establishment

Mineral nutrition	Sources
Nitrogen Excess	Aranjuelo 2014; Brockwell 1995; Serraj 1999; Singh and Usha 2003; Walley et al. 2005; Zahran 1999
Micronutrient Deficiency	Brockwell 1995; Gonzalez-Guerrero et al. 2014; Graham 1981; O'Hara 2001; O'Hara 2014; Yanni 1992
Phosphorus Deficiency	Bremer 1989; Brockwell 1995; Graham 1981; Hogh-Jensen 2002; Israel 1987; Jakobsen 1985; Leidi and Rodriguez Navarro 2000; McCauley 2011; O'Hara 2014; Zahran 1999
Abiotic factors	
Temperature (high or low)	Arayangkoon 1990; Bordeleau and Prevost 1994; Graham 1981; Hungria and Franco 1993; La Favre and Eaglesham 1986. O'Hara 2014; Zahran 1999
Water Stress (flood or drought)	Aranjuelo et al. 2014; Bremer 1988; Brockwell 1995; Graham 1981; Hunt 1981; Kirda 1989; O'Hara 2014; Serraj 1999; Venkateswarlu 1989; Williams and De Mallorca 1984; Zahran 1999
pH (acidic or alkaline)	Brockwell 1995; Graham 1981; O'Hara 2014; Rice 2000, Schubert 1990; Zahran 1999
Salinity	Azcon and El-Atrash 1997; Borderlau and Prevost 1994; Rao 2002; Zahran 1991; Zahran 1999
Toxicity	
Heavy metal contamination	O'Hara 2014; Wani 2007; Zahran 1999
Toxic seed pelleting	Brockwell et al. 1995; Deaker 2004; O'Hara 2014
Pesticide	Brockwell et al. 1995; Graham 1981; O'Hara 2014; Zahran 1999
Biotic factors	
Competitive native microorganisms	Brockwell 1995; Kyei-Boahen 2017; O'Hara 2014; Requena 1997
Poor inoculant adhesion and survival	Deaker 2004; Elegba and Rennie 1984; Hoben 1991; O'Hara 2014

Table 2.2 Field Conditions and Nutrient Solutions

Soil Type	Willacy fine sandy loam			
Soil pH	8.1			
Nutrient	**Field Conditions (ppm)**	**Concentration**	**Soil Impact (µg/g dry soil)**	**% Field Level**
Micronutrient				
Boron	0.79	25 µM H_3BO_3	0.68	86.1
Cobalt	0.04	1.7 µM $CoCl_2$	0.25	625
Copper	0.34	0.5 µM $CuSO_4$	0.08	23.5
Manganese	5.27	2 µM $MnCl_2$	0.28	5.3
Molybdenum	0.01	0.5 µM Na_2MoO_4	0.12	1200
Zinc	1.34	2 µM $ZnSO_4$	0.33	24.6
Phosphorus				
High	59.2	2 mM KH_2PO_4	155	262
Low	59.2	0.1 mM KH_2PO_4	7 (P)	12
		1.9 mM KCl	175 (K)	
Field	59.2	2 mM KCl	173	
Nitrogen				
High	18.8	5 mM CaN_2O_6	624	3319
Field	18.8	5 mM $CaCl_2$	410	
Low	18.8	5 mM $CaCl_2$	422	
All chemicals from Sigma-Aldrich, St. Louis, MO				

Table 2.3 Nodule and Plant Indicators for Moisture, Micronutrient, and Sterilization Experiments

	Moisture			**Micronutrient**		**Sterilization**	
	High	Mid	Cycle	Mi+	Mi-	S+	S-
Nodule data							
Number	23 a	17 a	28 a	22 a	17 a	18 a	18 a
Biomass (g)	0.80 a	0.56 ab	0.31 b	1.02 a	0.56 b	0.08 a	0.70 b
Activity (%)	64 a	55 a	63 a	56 a	55 a	18 a	60 b
Nodule: Plant Ratio (%)	5.35 a	4.71 ab	3.65 b	6.49 a	4.71 b	1.83 a	5.09 b
Plant data							
Max photosynthesis	9.91 a	9.71 a	7.24 a	7.99 a	9.71 a	3.05 a	8.61 b
Leaf area (cm^2)	399.3 a	321.0 a	260.7 a	426.6 a	321.0 a	79.6 a	368.9 b
Root (g)	0.43 a	0.26 b	0.23 b	0.39 a	0.26 b	0.20 a	0.31 b
Stem (g)	1.37 a	1.14 a	0.88 a	1.57 a	1.14 a	0.63 a	1.32 b
Leaf (g)	1.08 a	0.78 ab	0.64 b	1.06 a	0.78 a	0.23 a	0.93 b
Total (g)	2.88 a	2.19 ab	1.75 b	3.01 a	2.19 a	1.07 a	2.56 b
Root: Shoot Ratio (%)	17.95 a	15.02 a	15.65 a	15.02 a	15.02 a	25.71 a	14.26 b
* Moisture, Micronutrient, and Sterilization experiments were analyzed separately. For each experiment, means within a row that share a letter are not significantly different (α=0.05).							

Table 2.4 Nodule and Plant Indicators for Phosphorus and Nitrogen Experiments

	Phosphorus			Nitrogen		
	Field P	Low P	High P	Below field N	Field N	High N
Nodule data						
Number	18 a	13 a	25 a	15 a	15 a	0 b
Biomass (g)	0.51 a	0.64 a	0.75 a	0.32 a	0.39 a	0.00 b
Activity (%)	45 ab	33 a	60 b	31 a	44 a	0 b
Nodule: Plant Ratio (%)	5.48 a	5.72 a	6.25 a	5.68 a	4.32 b	0.00 c
Plant data						
Max photosynthesis	7.98 a	7.27 a	7.01 a	4.59 a	7.02 a	6.76 a
Leaf area (cm^2)	252.3 a	287.5 a	330.2 a	137.9 a	230.6 ab	309.0 b
Root (g)	0.24 a	0.25 a	0.26 a	0.17 a	0.23 a	0.17 a
Stem (g)	0.87 a	1.02 a	1.16 a	0.66 a	0.88 a	1.04 a
Leaf (g)	0.64 a	0.71 a	0.82 a	0.39 a	0.63 ab	0.87 b
Total (g)	1.75 a	1.98 a	2.24 a	1.21 a	1.74 ab	2.09 b
Root: Shoot Ratio (%)	15.36 a	16.29 a	14.49 a	16.80 a	15.79 a	9.97 b

*Phosphorous and Nitrogen experiments had independent controls and were analyzed separately. For each experiment, treatments which share a letter did not significantly differ (α=0.05)..

Table 2.5 Nodule and Plant Indicators for Sterilization Experiment

	Sterilization					
	S+	S-	M+	M-	R+	R-
Nodule data						
Number	18	18	**21**	**14**	**22**	**13**
Biomass (g)	**0.08**	**0.70**	0.44	0.33	0.33	0.44
Activity (%)	**18**	**60**	43	34	39	39
Nodule: Plant Ratio (%)	**1.83**	**5.09**	4.08	3.29	3.50	3.42
Plant data						
Max photosynthesis	**3.05**	**8.61**	6.08	5.58	6.53	5.13
Leaf area (cm^2)	**79.6**	**368.9**	243.1	205.5	200.4	248.1
Root (g)	**0.20**	**0.31**	0.29	0.23	0.23	0.28
Stem (g)	**0.63**	**1.32**	1.08	0.87	0.89	1.06
Leaf (g)	**0.23**	**0.93**	0.63	0.53	0.51	0.65
Total (g)	**1.07**	**2.56**	2.00	1.63	1.64	1.99
Root: Shoot Ratio (%)	**25.71**	**14.26**	19.26	20.70	20.47	19.50

*Results were compared among pairs (S+/S-; M+/M-; R+/R-). Statistically significant differences (α=0.05) shown in bold.

Table 2.6 Pearson Correlation Reports for Nodule Biomass vs Total Plant Biomass

Treatment	Correlation Coefficient	P value	N
Moisture	0.853	2.2E-09	30
Micronutrients	0.846	0.00000133	21
Phosphorus	0.832	6.49E-08	31
Nitrogen	0.0963	0.606	31
Sterilization	0.929	7.19E-19	42
Overall	**0.771**	**8.26E-28**	**135**

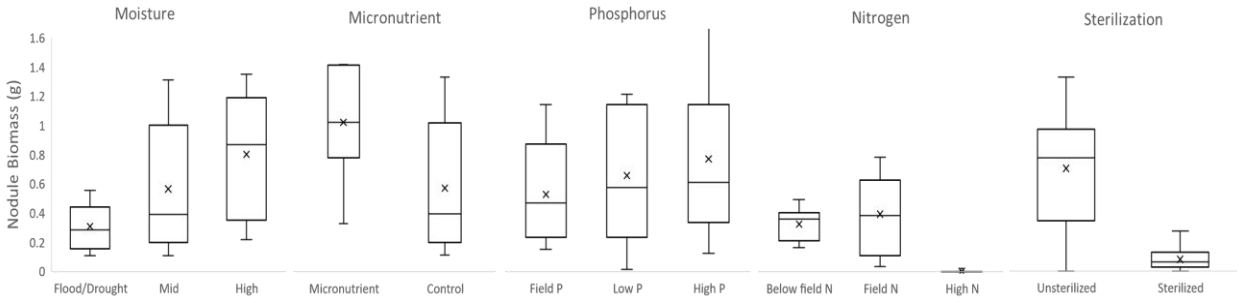


Figure 2.1 Nodule Biomass Plot

All experiments except phosphorus showed significant differences in nodule biomass. High moisture significantly increased nodule biomass over flood/drought cycle, while mid-range moisture significantly differed from neither high nor flood/drought. Micronutrient addition increased nodule biomass while nitrogen addition completely inhibited it. There was no significant difference between mean nodule biomass field N and low N. Soil sterilization significantly impeded nodule formation.

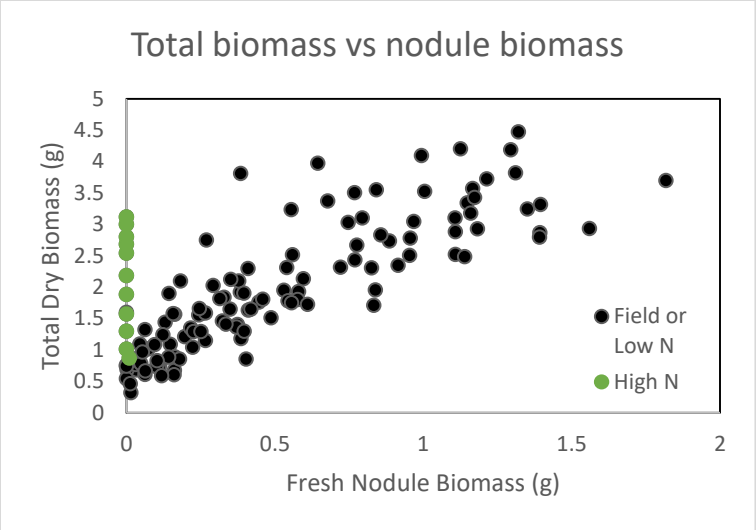


Figure 2.2 Correlation between Nodule Biomass and Total Plant Biomass:

Positive correlation between nodule biomass and total plant biomass ($r=0.771$, $p<0.001$), Notable outliers (green) are high nitrogen treatment which did not produce nodules. Their plant biomass was independent of nodule biomass. Additional correlation values for each of the treatment subsets shown in Table 2.6.

CHAPTER III

THE ROLES OF BORON, COBALT, COPPER, MANGANESE, MOLYBDENUM, AND ZINC IN NODULATION

Abstract

When they are effective, leguminous cover crops are a valuable tool for sustainable nutrient management. However, the symbiotic partnership between legumes and nitrogen fixing rhizobia is vulnerable to several abiotic and biotic stressors that reduce nitrogen fixation efficiency in real world contexts. One such limitation is micronutrient availability. The rhizobium symbiosis requires micronutrients including boron, cobalt, copper, molybdenum, manganese, and zinc (among others), sometimes at higher rates than the free-living plant or bacteria alone. Previous work investigating nodulation failure in a large cover crop trial in subtropical south Texas suggested that the addition of a micronutrient solution including B, Co, Cu, Mo, Mn, and Zn could significantly increase nodule biomass and other plant health indicators. This experiment was designed to clarify which of the six micronutrients were most active in the observed improvements and to inform potential efforts to improve nitrogen fixation efficiency. In this experiment, however, no significant difference was observed for nodule biomass or any other plant or nodule indicator between any of the micronutrient treatments and the control. This unexpected result may have been influenced by discrepancies in plant stress between the two experiments and the important role micronutrients play as stress mediators.

Introduction

Cover crop implementation is on the rise in the United States due to its potential for erosion reduction, increased organic matter, weed suppression, disease and pest cycle disruption, nutrient management, and other benefits (Snapp et al. 2005). Many farmers utilizing cover crops are interested in potential nitrogen gains from leguminous cover crops like vetch, clover, cowpea, and sunn hemp (Sarrantonio and Gallandt 2003). Few cover crop proponents, though, discuss the possibility that leguminous cover crops might fail to form nodules and provide expected nutrient benefits. This potential pitfall emerged when 14 acres of leguminous cover crops failed to form nodules during a winter field trial in subtropical south Texas. However, examples of nodulation failure are widespread and have been noted by researchers regularly over the years (Date 1970; Graham 1981; Brockwell et al. 1995).

Even under ideal nitrogen fixation circumstances the economic benefits of legume cover crops are not always clear (Sarrantonio and Gallandt 2003). When only nitrogen fertilizer replacement value is considered (rather than potential long-term benefits), the high cost of legume seeds can outweigh the low cost of synthetic nitrogen fertilizer (Mallory et al. 1998). Replacing synthetic nitrogen inputs with biological nitrogen from legumes has ecological benefits, such as reduced nitrous oxide emissions and lower nutrient runoff (Vitousek et al. 1997; Crews and Peoples 2004), but on-farm implementation depends on the cost-effectiveness of this strategy. If efficient nitrogen fixation cannot be assured, it may be a better alternative for farmers to prioritize other benefits of cover cropping using non-legume options with cheaper seeds. Farmers require better information on this topic to make sustainable and cost-effective decisions on their land.

Successful biological nitrogen fixation rests on a delicate partnership between rhizobia and their legume host, and any factor that impacts the health and growth of either symbiotic partner can impact nitrogen fixation efficiency (Brockwell et al. 1995). Micronutrient deficiencies are one factor that can reduce nodulation and nitrogen fixation. Research on this topic typically investigates one micronutrient and its impact on one legume species rather than the complex interactive effects of multiple micronutrients. Even comprehensive reviews seem unable to pinpoint micronutrient thresholds in the soil for effective nodulation and nitrogen fixation, a serious barrier to field applications of this information (O'Hara et al. 1988; O'Hara 2001).

Essential nutrients are elements that an organism's metabolism requires to complete its lifecycle and that cannot be replaced by some other element (Fageria et al. 2002). Cobalt, copper, manganese, molybdenum, and zinc are all essential legume nutrients that have also been identified as key micronutrients for rhizobia metabolism. Boron, although not required by rhizobia, is an essential plant nutrient and is involved in the formation of the legume-rhizobia symbiosis (O'Hara 2001). Rhizobia are encased within plant tissue and rely entirely on the nutrients that the plant releases to them (Dilworth and Glen 1984). Therefore, deficiencies in plant nutrition will likewise be passed on to the resident rhizobia.

In addition to the direct metabolic impacts of micronutrient deficiency on rhizobia, micronutrients can indirectly impact nodule formation and activity through several other pathways (Smith 2002). Micronutrient nutrition can increase plant resistance to biotic and abiotic stressors, like pathogens, drought and high temperatures (Hajiboland 2012). These stressors increase reactive oxygen species (ROS) levels in plants. ROS are a powerful signaling pathway in plant responses to pathogens and stressors but also cause oxidative damage if they are not

detoxified (Apel and Hirt 2004). One proposed explanation for the connection between micronutrient nutrition and stress resistance is that micronutrients are common components of the enzymes responsible for scavenging and detoxifying ROS (Rubio et al. 2007).

Boron

Legumes tend to be more susceptible to B deficiency than grasses and other crops (Gupta et al. 2008). Under B-deficient conditions, nodule formation can be severely limited or eliminated (O'Hara et al. 1988). B helps maintain nodule cell wall structure, serves as a signaling agent during the early phases of plant/bacteria communication, and modulates the infection thread through which rhizobia colonize the plant cell (Bolanos et al. 2004). Controlled studies on soybean have confirmed that B deficiency significantly impacts nodule development and weight, but mid-season withdrawal of B does not change nitrogen fixation activity (Yamagishi and Yamamoto 1994).

Cobalt

Although not essential for the growth of most plants, Co is an essential nutrient of rhizobia and is a requirement of nitrogen fixation (Fageria et al. 2002). Plants grown under Co deficiency often have similar levels of nodule tissue, but that tissue has lower bacteria counts and leghemoglobin concentrations (Riley and Dilworth 1984). Leghemoglobin, which is responsible for the distinctive pink color inside active nodules, is an important compound for nitrogen fixation because it binds free oxygen. This is important because the nitrogenase enzyme is ineffective in the presence of oxygen (Appleby 1984).

Copper

Plants and rhizobia require similar amounts of Cu for effective growth and function (Snowball and Robson 1980). Cu deficiency can decrease rhizobia concentrations, increase

nodule starch accumulation, and alter nodule growth patterns (Cartwright and Hallsworth 1970). Cu-deficient plants have also shown lower levels of iron, nodule biomass, and nodule leghemoglobin content (Seliga 1993). Cu is linked to molybdenum metabolism due to its involvement in molybdenum cofactor biosynthesis (Hansch and Mendel 2009). Finally, Cu is a component of superoxide dismutase, an antioxidant enzyme that protects against oxidative damage from ROS compounds like superoxide radicals and H_2O_2. Nodules produce ROS at several stages in the nitrogen fixation process, so effective antioxidants are important to maintain effective nodule function (Rubio et al. 2007).

Manganese

Although manganese has a documented role during the initial root infection process (Kijne et al. 1988; Gonzales-Guerrero et al. 2014), it mostly impacts nitrogen fixation indirectly as a determinant of plant health (O'Hara 2001). Like Cu, Mn also plays a role in antioxidant enzymes that protect against oxidative damage. Superoxide dismutases (SODs) with Mn as a component are typically found within the rhizobia unlike Zn and Cu SODs which are in plant cytosol and plastids (Rubio et al. 2007). Mn is also involved in ureide breakdown. Ureide is the form of nitrogen exported by the nodules of many tropical legumes including cowpeas (Unkovich et al. 2008). When nitrogen fixation is limited by ureide build-up, as often occurs under drought stress, supplemental Mn has been demonstrated to alleviate ureide accumulation and restart nitrogen fixation (Purcell et al. 2000; Sinclair et al. 2003).

Molybdenum

Mo is a fundamental component of nitrogenase, the enzyme responsible for nitrogen fixation (O'Hara 2001; Seefeldt et al. 2009). Although Mo deficiency reduces rhizobial reproduction and impedes nitrogenase activity, it does not typically play a role in nodule

initiation (Jongruaysup et al. 1993). Under Mo constraint, nodules still form, sometimes in greater quantities than on plants with adequate Mo (O'Hara 2001). Some legumes, like black gram (*Vigna mungo*), accumulate Mo in their seeds to a level that meets the nutritional needs of the subsequent plant, even when grown in Mo-deficient soil (Jongruaysup et al. 1997). When Mo deficiency does occur, plants show signs of nitrogen deficiency due to Mo's central role in nitrogen fixation (O'Hara et al. 1988).

Zinc

Plants with adequate Zn nutrition have greater nodule numbers, biomass, leghemoglobin content, N fixation, and plant biomass than Zn deficient plants (Shukla and Yadav 1982; Demetrio et al. 1972). Although not active in nodule formation, Zn is important to bacterial reproduction because Zn is a component of enzymes involved in DNA replication (Hansch and Mendel 2009). Zn, like Cu and Mn, is also involved in plant antioxidant enzymes (Rubio et al. 2007). It is difficult to pinpoint optimal zinc levels for nitrogen fixation because wide ranges of Zn requirements have been documented across rhizobial strains, ranging between 0.1 and 1.0/µM Zn. Most of these studies have been conducted in liquid growth media with few explorations of optimal soil levels (O'Hara et al. 1988).

Previous work on this topic (Chapter II) suggested that micronutrient levels might be a significant determinant of nodulation success in the sandy loams of the Rio Grande Valley. In the initial experiment, micronutrient addition increased nodule biomass by 79 percent over field soil. However, in this prior work, six micronutrients (boron, cobalt, copper, manganese, molybdenum, and zinc) were added simultaneously in a single micronutrient solution. The original design did not enable distinctions among the contributions of each micronutrient.

This experiment revisits micronutrient impact on nodulation to better identify which micronutrient(s) actively contributed to observed improvements. This follow-up examined the impact of micronutrient additions of B, Co, Cu, Mn, Mo, and Zn in isolation on nodule formation in cowpea (*Vigna unguiculata*), a common leguminous cover crop. We expected to similar increases in nodule tissue for one or more of the micronutrients added individually.

Methods

Iron and Clay cowpea seeds (*Vigna unguiculata;* Johnny's Seeds, Winslow, ME) were surface sterilized through immersion in 2% hypochlorite solution for five minutes, followed by five rinses with sterile water. Two seeds were then planted into each of 72 15 cm plastic pots with 1500 g of a 1:1 mixture of perlite and soil obtained from the field where nodulation failure occurred (Hilltop Gardens, Lyford, TX). Field conditions for this soil are shown in Table 3.1.

At planting, a 1 ml solution of rhizobium inoculant solution (2 g inoculant/500 mL water; Verdesian Guard-N®, Cary, NC) was applied to the seed. Pots were thinned to one plant each after 7 days. Cowpeas were randomly assigned to one of 8 treatments that were watered with a nutrient solution of B, Co, Cu, Mn, Mo, or Zn individually, all 6 micronutrients together, or tap water (control). Field conditions and nutrient solution additions are shown in Table 3.1. The plants were grown for 45 days in Percival Environmental Growth Chambers (Perry, Iowa) with 15 hours of light (PAR – 440 $\mu mol/m^2/s$) every 24 hours. Light period temperatures were 27 ^oC, dark period temperatures were 24 ^oC, and relative humidity ranged between 45 and 70%. Pots were watered with 150 mL of the designated nutrient solution every three days for a total of 16 waterings (2.4 L solution/plant). Each treatment had 9 replicates for a total of 72 individuals.

Data were collected 45 days after seeding from all 9 replicates for each treatment. Pre-termination measurements included spectral signatures (ASD Handheld 2, Malvern Panalytical,

Longmont, CO) and chlorophyll content (SPAD 502 Chlorophyll Meter, Spectrum, Aurora, IL). After termination, roots were cleaned and examined for nodules which were counted, weighed, and checked for internal color as an indicator of nitrogen fixation activity. Plants were then dried for at least 72 hours at 70^{o}C and the dry biomass of roots, stems, and leaves for each plant were recorded.

Where assumptions of normality and equal variances were met, one-way analyses of variance were used to examine differences among treatments. Multiple comparisons were performed using the Holm-Sidak method (SYSTAT™, San Jose, CA).

Results

Table 3.2 shows all nodule and plant parameters for each of the 8 micronutrient treatments. One-way ANOVAs showed no significant difference among the treatments at the 0.05 level of significance for nodule biomass ($F(7,64) = 1.686$, $p = 0.128$), chlorophyll content ($F(7,64) = 0.276$, $p = 0.961$), stem biomass ($F(7,64) = 1.639$, $p = 0.141$), leaf biomass ($F(7.64) = 1.709$. $p = 0.123$), total biomass ($F(7,64) = 1.828$, $p = 0.097$), root to shoot ratio ($F(7,64) = 1.255$, $p = 0.287$), and nodule to total biomass ratio ($F(7,64) = 0.944$, $p = 0.479$).

Data for the remaining variables failed tests of normality so Kruskal-Wallis ANOVA on ranks test was employed. No significant impacts of micronutrient application were detected for nodule number ($H(7) = 9.688$, $p = 0.207$), nodule activity ($H(7) = 10.556$, $p = 0.159$), nor root biomass ($H(7) = 9,099$, $p = 0.246$).

Discussion

Previous work in this soil showed that micronutrient additions could increase nodule biomass by 79% over field soil and improve other measures of plant health. This experiment was expected to show similar results for at least one of the micronutrients added individually. This

was not the case and no significant differences were detected among the treatments for any of the measured indicators of nodulation success and plant health.

Toxicity

Micronutrients, as critical as they are to plant health in small quantities, are also heavy metals that can cause severe toxicity problems for plants in excess. Possible toxicity was considered as a potential explanation for the unexpected results of this experiment, due to the slight reductions in plant growth and nodule biomass for some micronutrient treatments like Cu and Zn. However, toxicity is unlikely for several reasons. First, no plants in the study displayed any traditional signs of micronutrient toxicity (e.g. interveinal necrosis in high-B plants, chlorosis, shoot stunting and dense barbed wire like roots in high Cu and Zn plants, and striped pale leaves in high Co plants) (Fageria et al. 2002). Many toxicity problems are evident in leaf yellowing, necrosis, and chlorosis, all of which should be detectable by chlorophyll content (Rahman et al. 2004; Hernandez-Apaolaza 2014). There were no significant differences across mean chlorophyll content for any of the treatments. Zn and Cu, although lower than other treatments for nodule biomass and plant biomass, displayed slightly higher chlorophyll content.

Additionally, if the non-significant biomass decreases in the Zn and Cu treatments were related to toxicity, similar declines should also have been evident in the cowpeas that received all six micronutrients together. The six-micronutrient treatment received the same total amount of each micronutrient as each of the single micronutrient treatments. The six-micronutrient plants had similar nodule biomass and higher total plant biomass than the control. These results would be unlikely if the plants were suffering from toxic micronutrient levels.

The micronutrient amounts applied in initial greenhouse experiment were slightly higher than in this experiment due to the longer duration (75 days vs 45 days). If toxicity concerns were

legitimate, the plants in the greenhouse experiment receiving higher doses should also have demonstrated symptoms of toxicity. For these reasons, toxicity can safely be eliminated from consideration.

Insufficient Treatment Impact

Based on the impact of micronutrient additions compared to its field level presence (Table 3.1), certain micronutrient additions may have been too small to see the expected effects. The experiment was designed with nutrient solutions of a certain molar concentration in mind. Due to the challenges in finding specific micronutrient recommendations, the nutrient concentrations were based instead on a modified Hoagland solution (Taiz et al. 2015). Molar concentrations were converted retrospectively to µg nutrient/g of dry soil to estimate their effect on the native soil and then compared to the initial field level for each micronutrient.

In hindsight, these nutrient levels should have been chosen with more attention to ambient field concentrations for these micronutrients in mind. Instead, the impact of these treatments ranged from a miniature 3% increase over field level (Mn) up to a 800% increase (Mo). Other field impacts included 400% (Co), 54% (B), 15.7% (Zn) and 14.7% (Cu).

Micronutrients can be a critical component of plant health and legume success, but they are particularly challenging for farmers to conceptualize and manage effectively. Choosing the appropriate soil test to determine plant-accessible levels of micronutrients is an important step in this process. Tests that involve moderate to intense acid digestions may not be the best indicator of plant accessible nutrients (McLaughlin et al. 1999).

Although they are physically present in the soil, plants only have access to nutrients that are soluble by water or the mild carbonic acids released by their roots (Garcia 2017). Soil pH also highly influences the solubility, mobility, and plant availability of micronutrients. Generally,

the availability of Mo increases with pH increases, while availability of B, Cu, Co, Mn, and Zn decline in more alkaline soils (Fageria et al. 2002). Soil pH may have been a relevant factor in the alkaline soil where the initial nodulation failure occurred (Table 3.2). It is important for farmers who are interested in managing their soil nutrition more effectively to be able to consult with experts in soil chemistry who are familiar with the constraints and conditions of their soil type.

Inconsistent Experimental Conditions

This experiment was conducted based on the results of a previous study and was meant to clarify the micronutrient portion of those results. Instead, the results here directly contradict the conclusion that micronutrients significantly impact nodule and plant biomass. Since these two studies are interconnected and the second study grew organically out of the first, it is tempting to dwell on the comparisons between them and their contradictory results. However, there were several major differences between these two experiments that make it inappropriate to consider them exact parallels.

First, the initial greenhouse experiment ran for 75 days while the growth chamber experiment was only conducted for 45. This represents a 40% cut in experiment duration which was accompanied by a corresponding 36% drop in total volume of nutrient solution applied. The plants in the growth chamber experiment therefore received an overall lower total addition of micronutrients than their greenhouse counterparts. The differences in location also produced some major differences in environmental conditions which were ultimately evident in overall plant health. The temperatures inside the greenhouse were hotter and more variable and light quality was low and inconsistent, when compared with the controlled conditions of the growth chamber (Figure 3.2). All these added stressors in the greenhouse environment produced plants

that were in visibly poor health when compared to the plants from the growth chamber. Greenhouse plants were more likely to have leaf yellowing, elongated stems, and stunted roots while growth chamber plants much more closely resembled the growth pattern observed in field-grown cowpeas.

Research suggests that micronutrients' role in plant health becomes even more critical during periods of abiotic stress and adverse environmental conditions (Ashraf et al. 2012). It is possible that the added heat and light stress faced by the greenhouse plants combined with the higher micronutrient dosage received by these plants created a genuinely significant effect of micronutrient addition in the initial experiment. On the other hand, perhaps the growth chamber experiment with its more tightly controlled conditions and nearly double the replicates per treatment represents a more realistic picture of the impact of micronutrients on nodulation in this soil and the initial statistical significance in the greenhouse experiment was a fluke on low replicates and high variance. Whatever the case, the differences between the two experiments are substantial enough that no strong conclusions can be drawn from cross experimental comparisons.

Tables and Figures

Table 3.1 – Field Soil Conditions, Nutrient Solutions, and Treatment Impacts

Nutrients	*Field conditions (ppm)*	*Nutrient Concentration*	*Treatment impact (µg/g dry soil)*	*% Field Level*
Zinc[2]	1.34	2µM $ZnSO_4$	0.21	15.7
Copper[2]	0.34	0.5 µM $CuSO_4$	0.05	14.7
Cobalt[2]	0.04	1.7 µM $CoCl_2$	0.16	400
Molybdenum[2]	0.01	0.5 µM Na_2MoO_4	0.08	800
Boron[1]	0.79	25µM H_3BO_3	0.43	54.4
Manganese[2]	5.27	2µM $MnCl_2$	0.18	3.4

[1] Hot water , [2] DTPA
*All 6 micronutrient treatment received a combination of Zn, Cu, Co, Mo, B, and Mn at the same concentrations listed.

Table 3.2 Nodule and Plant Indicators

	All 6	B	Co	Cu	Mn	Mo	Zn	Control
Nodule data								
Number	33	32	31	13	24	22	21	36
Biomass (g)	0.59	0.65	0.45	0.21	0.52	0.49	0.33	0.64
Activity (%)	83.3%	91.2%	82.5%	91.7%	81.3%	97.2%	93.8%	79.7%
Nodule: Plant Ratio (%)	3.46	3.55	2.60	1.97	3.05	2.83	2.76	3.71
Plant data								
Chlorophyll content	39.2	36.2	39.3	39.7	39.4	38.6	41.4	38.1
Root (g)	0.91	1.01	0.77	0.54	0.78	0.73	0.63	0.75
Stem (g)	0.96	0.93	0.78	0.72	0.94	0.87	0.62	0.76
Leaf (g)	1.79	1.68	1.68	1.27	1.80	1.64	1.28	1.58
Total (g)	3.66	3.61	3.23	2.53	3.53	3.24	2.53	3.09
Root: Shoot Ratio (%)	33.3	38.9	31.3	26.8	28.6	29.4	33.4	30.5
* No significantly different results at the α=0.05 level for any of measured indicators.								

Table 3.3 Pearson Correlation Reports for Nodule Biomass vs Total Plant Biomass

	r	P value	N
Control	**0.787**	**0.0119**	9
All Micro	0.364	0.336	9
Boron	**0.728**	**0.026**	9
Cobalt	**0.734**	**0.0244**	9
Copper	0.265	0.491	9
Manganese	0.328	0.389	9
Molybdenum	**0.693**	**0.0385**	9
Zinc	0.355	0.349	9
Overall	**0.604**	**1.88E-08**	72

Figure 3.1 Correlation between Dry Nodule Biomass and Total Plant Biomass, by Micronutrient Treatment.

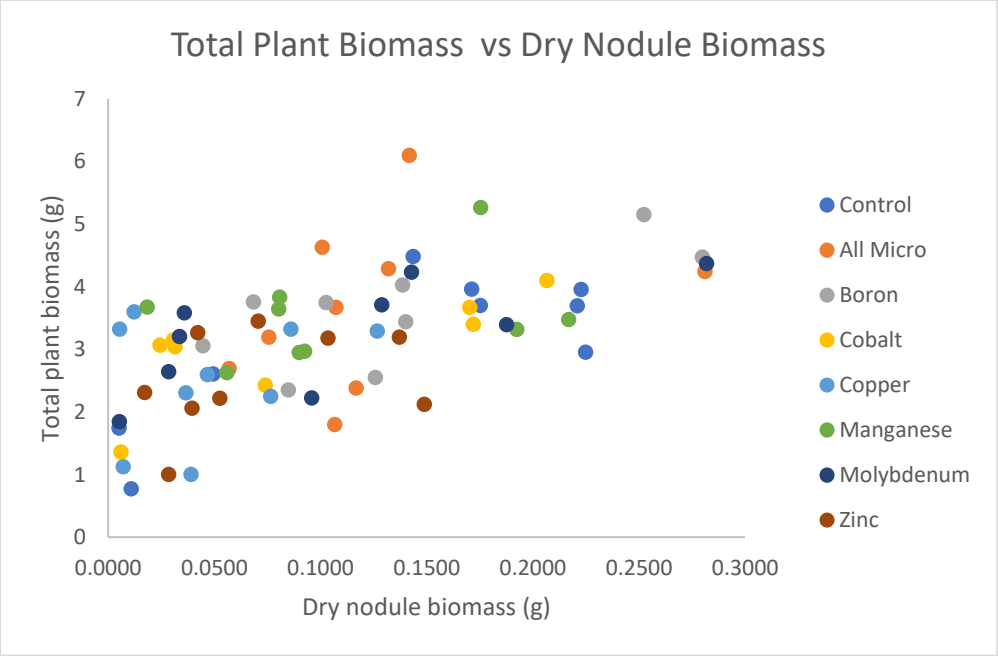


Figure 3.2 Light and Temperature Conditions for Growth Chamber and Greenhouse Experiments

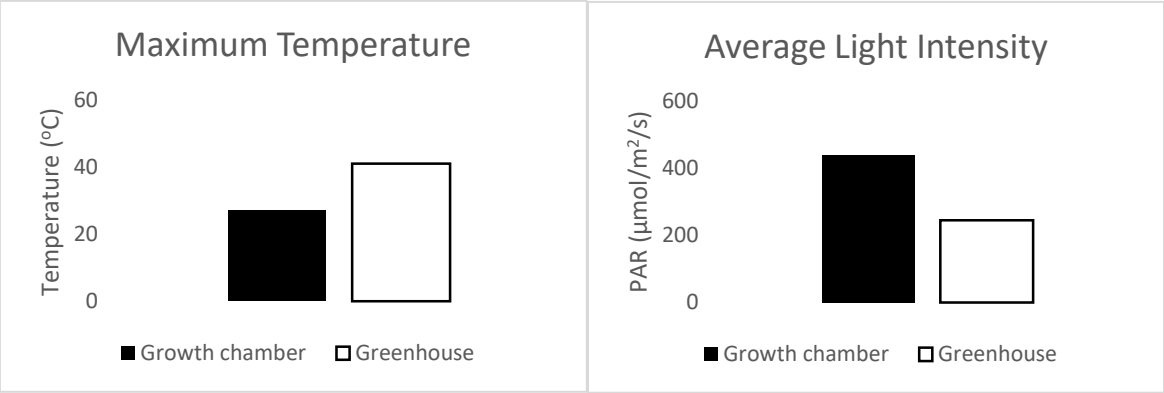


Figure 3.2 shows the discrepancies in temperature and light conditions between the growth chamber and greenhouse experiments. The plants in the greenhouse experienced less light (245 µmol/m^2/s) and higher temperatures (up to 41^{o}C) than their growth chamber counterparts (440 µmol/m^2/s, 27 ^{o}C). Differences in stress conditions between the two experiments could help explain their inconsistent results.

CHAPTER IV

CONCLUSION

Lessons, Setbacks, and Surprises

Leguminous Cover Crops Can Fail to Fix Nitrogen

This project began with an unwelcome surprise – 14 acres of unexpected nodulation failure. Although the limitations of biological nitrogen fixation are well-known among rhizobial biologists, the wider farming community is not as well-versed in the weaknesses of legumes (Zahran 1999; Brockwell et al. 1995). Since nodulation problems are not always visibly reflected in above ground plant health, growers must physically dig up plants and check for nodules to ensure a functional legume-rhizobia symbiosis. Unless farmers know more about potential nodulation problems, they are unlikely to diligently check for nodulation, especially in a cover crop where yield is not a primary concern. Researchers are working to develop technologies that could streamline field-scale nitrogen fixation estimates using remote sensing imagery (Thilakarathna and Raizada 2018). However, depending on the expense of these technologies, scouting in the field may remain the most accessible way for many small farmers to assess legume nodulation.

A Review of Suspected Nodulation Inhibitors

This study aimed to determine the most relevant factors in the nodulation failure at Hilltop Gardens and to consider potential interventions to improve nitrogen fixation results. Although these experiments were conducted in Hilltop's soil and the conclusions are most

relevant to the farm and field where the failure occurred, they have suggestive power for other regional farms as well. Relevant factors can be categorized into four groups: eliminated, confirmed, confused, or ignored.

Eliminated Factors – Nitrogen, Phosphorus, Mycorrhizae. Several of the proposed explanations for the nodulation failure and one proposed intervention can be eliminated. High nitrogen levels can inhibit nodule formation and nitrogenase activity (Serraj et al. 1999; Walley et al. 2005), but that was not a primary driver of limited nodulation in this case. Reducing the nitrogen content by half did not increase the number or weight of nodules over the field level soil. Similarly, phosphorus increases did not improve nodulation, thereby discounting phosphorous deficiency as an explanation. Other studies have suggested mycorrhizal inoculation as an effective intervention to improve nodulation, but the results of this experiment did not confirm their utility (Ortas 2003; Chalk et al. 2006). This technique might have more promise in soil lacking native mycorrhizae or if the inoculant includes more locally-adapted fungal strains.

Confirmed Factors – Moisture. The experimental results strongly suggest that increased moisture and increased frequency of watering improve nodulation outcomes. Unfortunately for dryland farmers, moisture limitation is particularly difficult to mitigate in the field. In a semi-arid region like the Rio Grande Valley, dry soils may consistently reduce the nitrogen fixation potential of leguminous cover crops, even when those soils remain moist enough for plants to survive. For farmers with irrigation access, it may not be cost-effective to water a crop that they do not intend to harvest. Cost-benefit calculations must be carefully considered, but legumes may provide a more consistent return on investment in regions that receive more regular rainfall.

Confused Factors – Micronutrients, Native Soil Communities. Micronutrient addition significantly increased nodulation in the greenhouse experiment, but the same was not observed in the follow-up growth chamber experiment. This discrepancy could have been caused by differences in stress faced by the two sets of cowpeas and the role of micronutrients in stress mediation in plants (Rubio et al. 2007; Hajiboland 2012). It was also difficult to determine micronutrient levels that were biologically significant without risking toxicity. This experiment could be repeated with higher micronutrient concentrations. However, in both experiments, cowpeas consistently formed nodules even at field level micronutrients. As an explanation for the failure of nodules to form in the field, micronutrients fall short.

The role of native soil microorganismal communities also remains unclear. Plants in sterilized soil performed markedly worse by all plant and nodule health indicators. This was true both for cowpeas that received rhizobial and mycorrhizal inoculation after sterilization and those that were uninoculated. Changes to the plant-available nutrient status (McCauley 2011) and populations of plant-growth-promoting rhizobacteria (Lugtenberg and Kamilova 2009) likely contributed but the data collected in this experiment was insufficient to fully untangle their impacts.

Ignored Factors – Rhizobial Inoculation. In hindsight, the set of factors investigated, while supported by the scientific literature, may have overcomplicated a simple problem – effective rhizobial inoculation. Nitrogen, phosphorus, moisture, micronutrients, and competitive soil microorganisms can all impact nodulation. However, the first requirement for nodule formation is that live rhizobia and viable legume seeds are present in the soil together. If rhizobia capable of infecting the legume species are neither present in the native soil nor introduced

through an inoculant, the legume-rhizobia symbiosis cannot form and even the best habitat and climatic conditions cannot mitigate this initial problem.

Although we cannot test the original rhizobial inoculant for viability (Lupwayi et al. 2000) or measure the number of rhizobia that survived on each seed (Materon and Weaver 1985) post-facto, other observations suggest that inoculation problems may have caused the nodulation failure. In the Fall 2018 field trial at Hilltop Gardens, 38% of the cowpea plants showed evidence of nodule formation. In the greenhouse and growth chamber experiments, 95% of plants formed at least one nodule (Figure 4.1). The scale of this improvement was unmatched by any of the other nodulation drivers under consideration.

Cowpeas in the greenhouse and growth chamber experiments were inoculated with a rhizobial slurry pipetted directly to the seed and surrounding soil while the cowpeas in the field were mixed with dry peat inoculant and water before planting by seed drill. Laboratory precision is hard to achieve in field inoculations, but improved strategies should be considered. Other methods to explore include granular and liquid inoculants (which typically require specialized equipment) and substances that can improve inoculant adherence like gum arabic, carboxymethyl cellulose, and vegetable oil (Elegba and Rennie 1984; Hoben et al. 1991; Deaker et al. 2004).

Regardless of inoculation method, sub-tropical and tropical legume species (sunn hemp and cowpea) have shown better nodulation success in the field than temperate legumes (clover, vetch, and pea), possibly due to the presence of native rhizobial colonizers for tropical legumes. Uninoculated cowpeas under field, green house, and growth chamber conditions will all form nodules in Hilltop soil, often at similar levels to their inoculated counterparts. Using legumes with native rhizobial partners could improve nodulation results. Burr medic, cowpea, common bean, and sunn hemp have all been observed to nodulate without inoculation in the Rio Grande

Valley. Confirming additional legume candidates with native rhizobia could help make nitrogen fixation success less reliant on inoculation. Searching for "promiscuous" legumes which can form nodules with multiple rhizobia species could also aid in this effort (Sprent 1989).

A Farmer's Guide to Maximizing Nitrogen Fixation

Problem-Driven Research and Accessible Results

As a farmer, it was important to me that my thesis research be driven by a real-world problem faced by farmers in the Rio Grande Valley. This project has helped me develop my skills as a "boundary spanner," someone who can stand at the border between information producers and information users and facilitate communication between the two in a way that is credible and useful to both (Safford et al. 2017). In the spirit of boundary spanning, the goal of this section is to condense the useful information I have gathered based on my research, readings, conversations with experts and personal agricultural experiences into a farmer-useful form.

How to Farm for Nodules: A Six-Step Process

Step 1 - Choose a legume that is likely to succeed. Legumes are the third largest plant family on earth with over 19,000 identified species that vary widely in their environmental tolerances (Christenhusz and Byng 2016). Only a small percentage of these legumes are grown agriculturally, but finding the best match for your season, soil, and climate can still be a challenging process. Temperate legumes prefer cooler temperatures and have some frost tolerance while sub-tropical or tropical legumes have low cold weather tolerance and perform better in warmer seasons. Table 4.1 shows some common cover crop candidates and their preferred climate. Legumes also vary in pH preferences, salinity tolerance, nutrient demands, and moisture requirements.

The local farming community can be a valuable source of advice on what legumes thrive or struggle in a region. Even if cover crops are new to the area, information on leguminous cash crops like soy can also be useful. Local information is usually more helpful than general extension advice from other regions. Once strong candidates are determined, small scale trials can help determine the best choice for farm-specific soil and micro-climate conditions.

Step 2 - Inoculate well with live rhizobia of the correct type. Effective inoculation requires a correct match between rhizobia and legume, live rhizobia, and good inoculation technique. Most legume species require a certain rhizobia species in order to nodulate. The leftover inoculant from a soybean planting, for instance, will not provide the rhizobia that a clover cover crop needs to nodulate. Commercial inoculants are usually labeled by their intended legume specie(s). Sometimes instead of a species, the inoculant will list an inoculant group letter. Inoculant groups for common cover crop species are listed in Table 4.2.

Rhizobial inoculants are a living product and it is important that they are stored under conditions that maximize the number of live bacteria. Inoculant viability can be damaged by temperatures below freezing or above 80 ^{o}F or by exposure to sunlight or toxic chemicals (O'Hara et al. 2014). Special consideration should be taken when shipping rhizobia, especially during the hot summer season, to make sure they are not exposed to temperature extremes. Once obtained, inoculants should be stored in a cool, dry place, used before their expiration dates, and applied to seeds within 24 hours of planting. Pre-inoculated seeds often show lower numbers of viable rhizobia and lower nodulation success rates due to rhizobial desiccation and antibacterial substances produced by some legume seed coats (Deaker et al. 2004).

Inoculation methods vary based on farm scale and available equipment, but the fundamental goal is always the same: to maximize the number of rhizobia stuck on each seed and

to keep those rhizobia stuck there until the seed is in the ground. On a small scale, inoculation equipment could be as simple as a plastic bag. Place the seeds in the bag with a little water and shake to wet the seeds. Then add powdered inoculant and shake again vigorously until the seeds are well coated. For medium scale farms, a cement mixer can be a helpful tool to ensure consistent rhizobial coating. Large scale farmers might consider investing in specialized equipment to allow for liquid or granular inoculant applications which sometimes show higher success rates. At any scale, careful attention to the inoculation step can help maximize nodulation and nitrogen fixation

Step 3 - Keep plants as healthy as possible. In a functioning nodule, the rhizobia are completely enclosed by legume root cells and entirely dependent on their plant host for carbohydrates, water, and essential nutrients (Dilworth and Glenn 1984). Any shortage or deficit that the plant experiences will be passed along to the nodules and nitrogen fixation rates will decline (Brockwell et al. 1995). Moisture deficits are particularly challenging to nitrogen fixation because the nodules lose water first when the plant is water-stressed (Serraj et al. 1999). Legumes perform best when they receive frequent, moderate moisture rather than infrequent flood/drought cycles.

Rhizobia trade plant accessible nitrogen for carbohydrates from their plant hosts, so anything that reduces a plant's photosynthetic capacity will also reduce its nitrogen fixation potential. Some other factors that can reduce photosynthesis rates include high temperatures, insufficient light, and plant diseases.

Step 4 - Check for nodules after 30 days. The time it takes for visible nodules to appear varies across legume species, but most cover crops will show signs of nodulation by three weeks after planting (Lindemann et al. 2015). Around one month after planting, a careful nodule farmer

should dig up some legume root systems, keeping them as intact as possible, to see what percentage of the plants have formed nodules, how many nodules each plant has, and what size the nodules are. More nodule tissue generally means more nitrogen fixation Take samples from multiple points across the field to avoid a sampling error from a spot that might not represent the entire field.

Step 5 - If nodules are present, estimate nitrogen availability for the subsequent crop, Farmers used to the precision of soil chemistry and specific fertilizer recommendations, the uncertainty of legumes can be concerning. How much nitrogen exactly did this cover crop provide? When will it be available for plant uptake? Are nitrogen levels sufficient for the next crop or should supplementary fertilizer be applied?

These questions have been a barrier to widespread adoption of biological nitrogen fixation as a major component of farm nutrient management. However, researchers at the University of Georgia have developed a nitrogen availability calculator to help answer some of these questions, available at http://aesl.ces.uga.edu/mineralization/. The calculator asks for information about cover crop species, termination/incorporation date, intended cash crop, target nitrogen fertilizer rate, termination method, dry cover crop biomass (lb/acre), and percent nitrogen, carbohydrate, cellulose, and lignin content in the residue.

This tool requires detailed records as well as laboratory crop residue analysis. This investment might not be a priority for every grower. However, with the requested information, the calculator can estimate the amount of nitrogen the cover crop will release two weeks and four weeks after termination. It also reports the estimated N credit or deficit for the next cash crop. This resource is tailored to the state of Georgia, but it can help estimate nitrogen availability for growers in other regions as well. The Georgia-specific part of this tool that it estimates residue

decomposition rates using county rainfall data. It will be most accurate for growers who can find a Georgia county with similar rainfall amounts to their farm in the target time frame. The developers hope to expand their coverage network to provide more accurate estimates in other southeastern states in the coming years (Gaskin et al. 2016).

Step 6 - If nodules are spotty or absent, troubleshoot with the nodulation flow chart. When nodule failure occurs, finding the root of the failure can help inform future cover cropping decisions. Some problems that prevent nodulation are easily remedied. Others might mean that leguminous cover crops are not the best investment for a certain farm context. Figure 4.2 provides a guide through the potential factors that can interfere with nodule formation. Potential interventions to improve nodulation are noted where relevant.

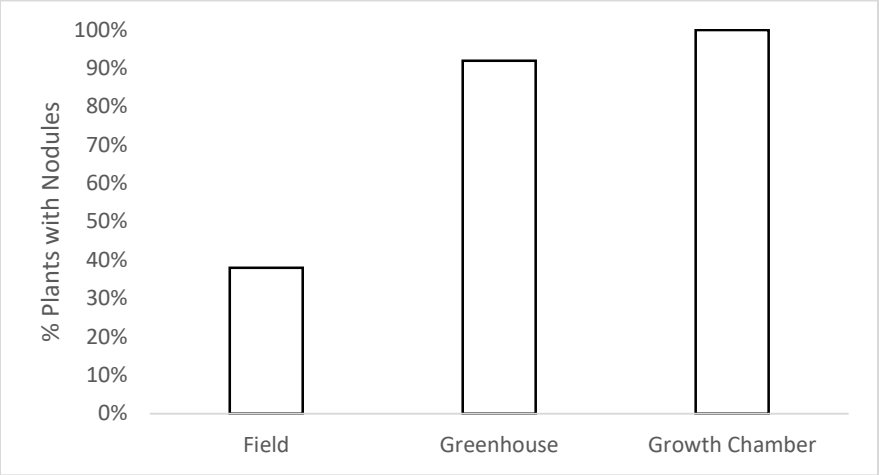
Tables and Figures

Table 4.1 Temperate and Tropical Leguminous Cover Crops

Temperate Legumes	
Common name	*Scientific name*
Clover	*Trifolium spp.*
Vetch	*Vicia spp.*
Pea	*Pisum sativum*
Lupine	*Lupinus spp.*
Medic	*Medicago spp.*
Lentil	*Lens culinarus*
Tropical Legumes	
Common name	*Scientific name*
Cowpea	*Vigna unguiculata*
Sunn hemp	*Crotalaria juncea*
Lablab	*Lablab purpureus*
Velvet bean	*Mucuna spp.*
Pigeon pea	*Cajanus cajan*
Soybean	*Glycine max*
Scarlet runner bean	*Phaseolus coccineus*
Mung bean	*Vigna radiata*
Perennial peanut	*Arachis glabrata*
Temperates adapted from Clark 2012. Tropicals adapted from Clark 2012 and Duncan 2017.	

Table 4.2 Inoculant Groups for Potential Cover Crops

Inoculant Group	**Legume Species (Common Name)**
A	Alfalfa, Medics (burr, barrel, snail, sphere, murex, strand, and disk), Lucerne
B	Clovers (white, red, strawberry, alsike, berseem, ball, suckling, talish)
C	Clovers (subterranean, balansa, crimson, purple, arrowleaf, rose, gland, helmet, Persian, bladder)
E	Field pea, Vetch (hairy, chickling, Narbon)
F	Faba bean, Lentil
G	Lupin
H	Soybean
I	Cowpea, Mungbean, Sunn hemp
J	Pigeon pea, Lablab, Horse gram
N	Chickpea
P	Peanut
S	Serradella (French, yellow)
Adapted from O'Hara et al. 2014	



Cowpeas in the greenhouse and growth chamber experiments which were inoculated with 1 mL of pipetted rhizobial slurry directly to the seed and surrounding soil showed a substantially higher nodulation rate than cowpeas in the field, inoculated in a more traditional way. The precise inoculation of controlled experiments cannot be replicated in the field, but other ways to bridge the gap and improve field inoculation outcomes must be considered.

Figure 4.1 – Cowpea Nodulation Rate, Field vs Laboratory

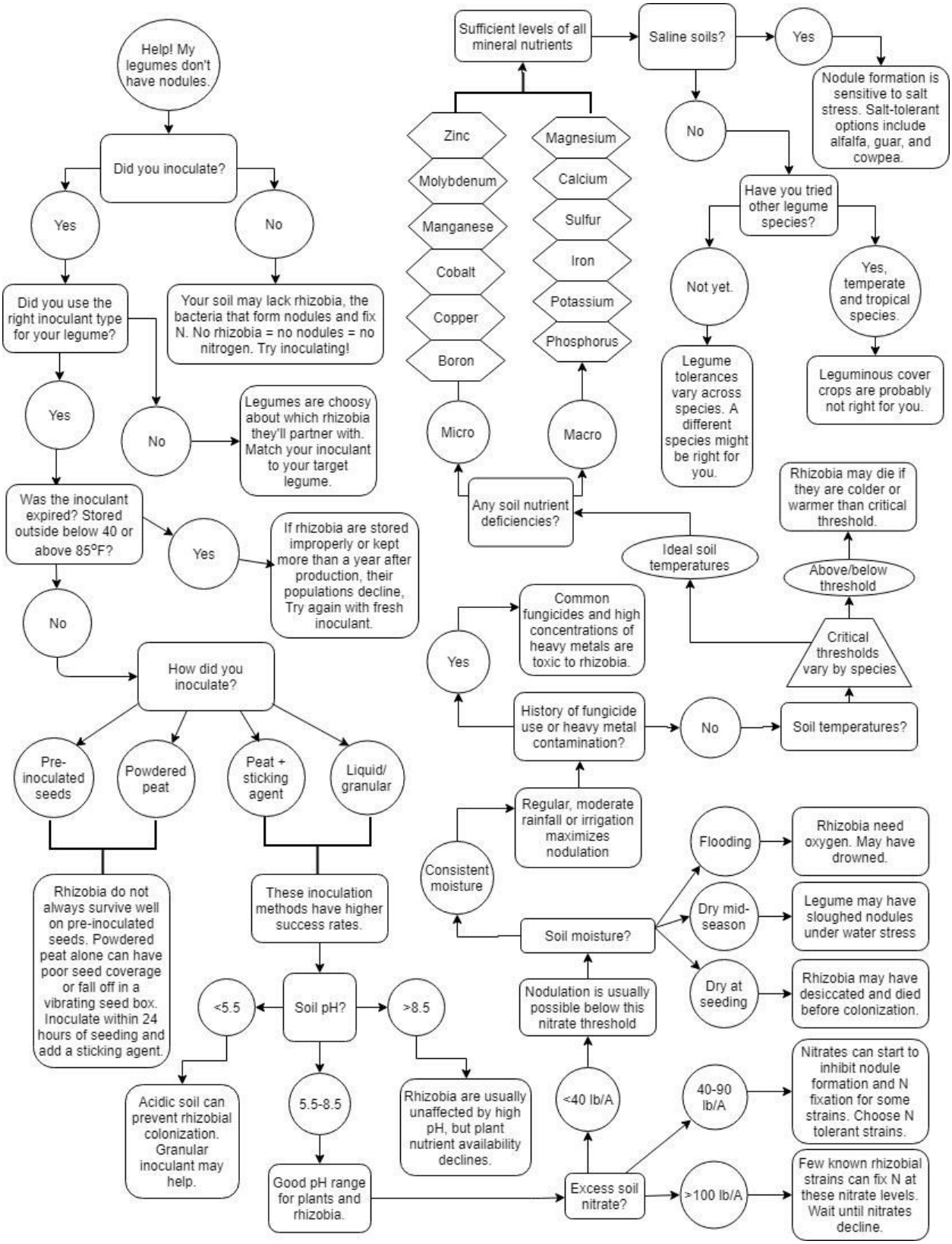


Figure 4.2 Troubleshooting Nodulation Failure

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BIOGRAPHICAL SKETCH

Stephanie L. Kasper graduated from Rhodes College in Memphis, Tennessee in 2014 with a B.A. in Anthropology and Sociology. A Victoria native, she returned to Texas to work on small sustainable farms closer to home. As the farm manager at Knopp Branch Farm in Edna, Texas, Stephanie realized how little geographically relevant information was available to help sustainable farmers in south Texas make smart economic and ecological decisions on their land. She took a farming sabbatical to learn more about the science of sustainable agriculture and participate in farmer-driven research projects in the Rio Grande Valley. She completed her Master of Science degree in Agricultural, Environmental, and Sustainability Sciences in May 2019. Stephanie can be contacted at stephanie.lynn.kasper@gmail.com.