EVALUATING FACTORS AFFECTING BIOLOGICAL NITROGEN FIXATION

IN LEGUMES PRODUCED IN THE SOUTHERN GREAT PLAINS

A Thesis

by

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ABSTRACT

Legumes grown as cash and cover crops are a valuable component of cropping systems. Legumes can fix N through a symbiotic relationship with root-associated bacteria, called Rhizobia, through a process called biological nitrogen fixation (BNF). In the Southern Great Plains region of the United States and particularly in Texas, there is very little information regarding the N-fixing potential of legumes. The research in this thesis aims to contribute information on BNF and associated plant and management factors for legumes grown in this region. The first study examined guar (*Cyamopsis tetragonoloba* L. Taub.), a legume produced in semi-arid regions around the world, primarily grown for industrial uses of the guar gum in its seed. The objective of this research was to test the efficacy of inoculants derived from four USDA Rhizobia strains listed as compatible with guar, as well as a mixture of all four strains, compared to un-inoculated plants in controlled greenhouse conditions. Among the tested strains, USDA 3089 and USDA 3386 had the greatest positive impacts, as they increased nodule weight, total assimilated nitrogen, biomass production, and protein production per plant relative to the un-inoculated control. These two Rhizobia strains were identified as beneficial and recommended for further testing in the field in development of an effective inoculant for guar. The second study also examined guar in a controlled environment, with the objective to provide initial understanding of temporal and spatial dynamics in root system physical and architectural traits of guar, including dynamics in nodule growth and senescence. Results illustrated the development of roots and nodules

over an 80-day period and within five depth ranges in a 130 cm tall soil column. The data showed that nodules were concentrated toward the soil surface and that the average nodule weight also decreased with soil depth. Root length density and root weight generally decreased with depth and root diameter increased with time. Guar's root architecture and its development over time is a novel addition to our knowledge of this drought-tolerant crop. The objective of the third study was to quantify BNF and productivity in four winter legume species (Austrian winter pea, crimson clover, hairy vetch, and lentil) grown as cash or cover crops in three distinct dryland cropping systems in the Southern Great Plains region. Austrian winter pea was among the best producers and seemed to have an advantage as a N-fixer in monoculture plantings due to its high %Ndfa, followed closely by hairy vetch. Results suggest that Austrian winter peas, guar, hairy vetch, and lentil can be incorporated into regional cropping systems to fix N, reducing the need for chemical fertilizer applications, while providing other ecosystem services.

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1. INTRODUCTION

1.1. Biological Nitrogen Fixation by Legumes

Legumes have played an essential role in agricultural production since the establishment of early civilization. They provide food for humans and fodder for domestic animals, in addition to providing many ecosystem services including erosion control, weed suppression, and contributing organic matter to the soil (Blesh, 2018; Drinkwater et al., 1998; Finney et al., 2017; Tonitto et al., 2006). Legumes, however, are most often incorporated into agricultural systems due to their ability to fix nitrogen in a process known as Biological Nitrogen Fixation (BNF) (McCauley, 2011). BNF is a process of fixing atmospheric N into a form that can be used by plants and microorganisms. Legumes form a symbiotic relationship with root-associated N-fixing bacteria called Rhizobia and incorporate fixed N into their biomass (McCauley, 2011).

Nitrogen is usually the most limiting nutrient in agronomic systems (Hungria and Vargas, 2000). In turn, commercial N fertilizer is the input most heavily relied upon in conventional cropping systems in the United States and the world (McCauley, 2011; Gruber and Galloway, 2008). Economic and environmental factors have led to concerns over the dependence of modern agriculture on synthetic fertilizers and a renewed interest in using alternative N sources (Huang, 2009; Tilman et al., 2001; Vance 2001). Incorporating legumes into cropping systems can reduce the need for added N fertilizer application by cycling fixed N from legume root exudates and legume residues into the system where it can become available for subsequent non-legume crops (Zetner et al.,

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2001). BNF is already a key component in organic systems by necessity and conventional system would benefit from an expansion of BNF as an alternative source of N (Blesh and Drinkwater, 2013; Connor, 2018; Miller et al., 2011).

1.2. Factors Affecting Biological Nitrogen Fixation in Legumes

There are several biological and environmental factors that affect BNF in legumes. These include soil constraints such as acidity, salinity, alkalinity, and nutrient deficiencies to name a few (Alexander, 1985; Bhardwaj, 1974; Bohlool et al., 1992; Liu et al., 2011). Studies have shown that plant nutritional status such as phosphorus and potassium levels affect legume BNF by controlling nodule growth and nitrogenase activity either directly or indirectly (Havelka et al., 1982; Liu et al., 2011). The concentration, and form of N already present in or applied to the soil also affects BNF in legumes. It has been widely reported that soil mineral N in the root zone inhibits legume nodulation, nodule establishment, and nitrogenase activity because it costs less energy for legumes to take up available N from the soil than to fix it biologically from the atmosphere (Abdel-Wahab, 1996; Cannell and Thornley, 2000; Hinson and Adams, 2020; Liu et al., 2011; Phillips, 1980; Purcell and Sinclair, 1990; Wood, 1996). Additionally, nodulation and nodule health are sensitive to drought and high temperature (Albrecht et al., 1984; Goh and Bruce, 2005; Liu et al., 2011; Roughley and Dart, 1970; Whitehead, 1995).

Symbiotic activity is majorly impacted by the presence, fitness, and compatibility of Rhizobium. Differences exist among species of Rhizobium in their ability to colonize

plant roots (Chatel and Greenwood, 1973). Studies have shown that competition among Rhizobium strains frequently results in a highly active strain being unable to compete with indigenous strains with low effectiveness (Alexander, 1985; Boonkerd et al., 1978). This presents a major practical problem when trying to inoculate a crop in a field where there may be indigenous strains of Rhizobia in the soil. Furthermore, both insufficient and excessive moisture, as well as exposure to low or high temperatures negatively affect the survival of rhizobia. Exposure of the soil to cycles of wetting followed by extensive drying, a common occurrence in semi-arid regions, reduces the viability of Rhizobium species and thereby reduces the capacity of associated legumes to form nodules and fix nitrogen (Pena-Cabriales and Alexander, 1979).

There are additional constraints to the optimal performance of BNF systems which include technical, socio-economic, and human-resource obstacles (Bohlool et al., 1992; Chianu et al., 2011). For example, the scale of inoculum production, the availability of suitable carrier material, and shelf-life of the finished product can be constraints to use of inoculants (Bohlool et al., 1992). Despite significant BNF benefits, farmers must consider field-level production constraints, implementation of new technology, and additional risks and costs involved when incorporating legumes into their cropping systems.

1.3. Legume-Based Biological Nitrogen Fixation in the U.S. Southern Great Plains

The Southern Great Plains includes parts of Kansas, Oklahoma, and Texas that are west of the 35-inch precipitation isohyetal identified under the Great Plains

Conservation Program (Soil Conservation Service, 1981). This region is characterized

by a sharp decrease in rainfall westward from the 100th meridian (Borchert, 1950) and

the predominant soils in this region are mollisols and alfisols (Soil Conservation Service,

1967). The total area of Kansas, Oklahoma, and Texas is about 268 million acres,

approximately two thirds of which are located in the Great Plains region. About 92% of

the land is undeveloped non-federal rural land and out of that, 7% is forestland, 11% is

pastureland, 29% is cropland, and 51% is rangeland (Anderson, 1995). The Texas

portion of the Southern Great Plains consists of the Texas Rolling Plains and Texas High

Plains subregions, semi-arid regions with major production of irrigated and dryland

crops (Modala et al., 2017). The two primary cash crops are cotton (*Gossypium hirsutum*

L.) and winter wheat (*Triticum aestivum* L.), while peanut (*Arachis hypogaea* L.), grain

sorghum, guar (*Cyamopsis tetragonolobus* L.), sesame (*Sesamum indicum* L.), and other

crops are also grown (Hinson and Adams, 2020).

In this region, wheat and rye are commonly used as cover crops in rotation with

major cash crops (Adhikari et al., 2017; DeLaune et al., 2012; Lascano et al., 2015), but

these systems lack BNF due to the lack of legume incorporation. A number of legume

species have been tested and shown to have potential in the Southern Great Plains and

could be incorporated into various agricultural systems in this region. Austrian winter

pea (*Pisum sativum subsp. arvense*), hairy vetch (*Vicia villosa*), lentil (*Lens culinaris*),

and other species are among the cool-season legumes that have been tested and are

grown to some extent by producers in this region as either forage in pasture-based

livestock production systems, as cash crops, or as cover crops (Adams et al., 2020; Guretzky et al., 2012; Sheaffer and Evers, 2007).

In forage-livestock systems, the decomposition of legume plant residue and the excretion of legume wastes by grazing animals are major components of cycling nutrients, particularly N (Rouquette and Smith, 2010). Research has been done to evaluate legumes, including guar, cowpea, soybean, and clovers for forage-based production systems focused on growing stocker cattle (Krenzer, 2000; Rao and Northup, 2009; Rouquette and Smith, 2010). Otuya (2019) assessed how legume forages such as alfalfa and yellow sweetclover served as alternative nutrient sources when interseeded with warm-season perennial grasses in the Texas High Plains. This research illustrated the effects of legume mixtures on total soil C, N, soil microbial biomass, and forage yield. Similarly, Cui et al (2014) and Bhandari et al (2020) assessed the role of interseeding legumes into grass to improve pasture soil health in the Texas High Plains. Both studies mainly illustrate soil parameters, but there is some information regarding N concentration and N contribution from the legumes over time. Other studies in Oklahoma evaluated legumes such as cowpea, alfalfa, and cicer milkvetch and found substantial residual N from legumes increased forage yield or quality for subsequent crops of switchgrass and wheat (Berg, 2008; Kandel et al., 2019; Sutradhar et al., 2017).

Legume cover crops improve soil health and farm management while fixing N and reducing the need for N fertilizer inputs. The Texas A&M AgriLife Extension Service established a project to demonstrate best management practices related to the management of nitrates through the use of winter cover crops in the Texas High Plains

and Texas Rolling Plains. Vetch was the only legume cover crop and authors reported biomass, amount of nitrogen per acre, and nitrate levels in the soil recycled after cover crop treatments (Dozier et al., 2008). A study in north Texas conducted an economic risk analysis to evaluate the profitability of tillage and cover crops (including a grass-legume mixture) in irrigated cotton production (Fan et al., 2020). The legumes in the mixture included vetch, clover, and pea, but the results only showed statistics of lint yield data from cotton production after each treatment and no information on legume productivity or BNF was presented. Keeling et al. (1996) evaluated 13 plantings of cool-season cover crops in the Texas High Plains and concluded that single species cover crops of rye, wheat, Austrian winter pea, and hairy vetch were all highly productive under dryland conditions. They reported higher biomass yields in wheat and rye than in legumes, but the authors did not investigate cover crop effects on BNF. Lewis et al. (2018) quantified the long-term impacts of conservation tillage and cover crop practices in the High Plains of Texas with a mixed species cover including rye, hairy vetch, radish, and winter pea. Results of this study contributed to knowledge of cotton production with regards to soil organic C storage, cotton lint yield, and economic returns, but did not look in depth at the legume productivity or nitrogen fixation.

1.4. Objectives

There are major gaps in knowledge regarding the N-fixing potential of legumes in Texas and regarding management of N-fixation in legumes grown in the region. As reviewed above, some studies in the Southern Great Plains have quantified the impact of

legumes in agronomic systems, but no research has been done to measure percent of N

derived from the atmosphere or to calculate total BNF in the region and few research

efforts have been made to improve our understanding of how to best manage legumes in

the region to optimize BNF. Therefore, the objectives of the research presented in this

thesis are to:

1. Test the efficacy of five Rhizobium inoculants on nodulation, nitrogen assimilation, and plant productivity of guar.
2. Develop a better understanding of spatial and temporal dynamics in root and nodule development in guar.
3. Directly quantify BNF and productivity in four winter legume species that are grown in the Southern Great Plains in three management systems.

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2. TESTING THE EFFICACY OF EXISTING USDA RHIZOBIA ACCESSIONS AS INOCULANTS FOR GUAR

2.1. Introduction

Guar (*Cyamopsis tetragonoloba* L.), which is also known as clusterbean, is an annual legume grown in semi-arid regions around the world (Gresta et al., 2013; Whistler and Hymowitz, 1979). This drought-tolerant plant is believed to have been domesticated from a wild species named *Cyamopsis senegalensis* in the geographic region of India and Pakistan, where a large fraction of global guar production remains today (Krishnan et al., 2011; Gresta et al., 2017; Mudgil et al., 2014; Whistler and Hymowitz, 1979). Guar is primarily grown today for the galactomannom gum (guar gum) extracted from the seed endosperm, which has many industrial uses as a stabilizer, emulsifier, lubricant, and thickener (Mudgil et al., 2014; Yadav et al., 2015). The use of guar gum was explored by several U.S. companies, which led to the development of a commercial industry for this product in 1955 (Abidi et al., 2015; Sharma and Gummagolmath, 2012). Today, guar gum is used in a variety of products in the food, textile, pharmaceutical, explosive, and oil and gas industries (Mudgil et al., 2014, Yadav et al., 2015).

In addition to its economic potential, guar provides added value in ecosystem services. As a legume, guar can associate with Rhizobia bacteria in the soil to form root nodules and fix atmospheric nitrogen, thereby increasing soil and crop fertility (Chaudhary and Sindhu, 2016). Guar is among the most drought and heat tolerant of all

crop species and has the ability to form root nodules at higher temperatures and in drier conditions than most legumes, making it highly valuable as a nitrogen-fixing crop for semi-arid regions (Gresta et al., 2013; Stafford and McMichael, 1991; Zahran, 1999). The salinity and alkalinity tolerance of guar adds to this functionality (Ashraf et al., 2002; Francois et al., 1990; Suthar et al., 2018; Undersander et al., 1991). Research on nodulation and nitrogen fixation in guar is limited but has included both agronomic and controlled-environment studies. A common theme in these published works is a perception that guar often does not nodulate effectively in field conditions (Abidi et al., 2015; Khandelwal and Sindhu, 2012).

Bhardwaj (1974) made observations of root nodulation in guar in the field with indigenous Rhizobium species and reported that 32% of guar plants had nodules, which was considered "very poor" when compared to nodulation rate in other legume species. Stafford and Lewis (1980) reported that inoculated guar produced an average of 36% more nodules per plant when compared with non-inoculated guar of the same variety. Khandelwal and Sindhu (2012) isolated 95 strains of Bradyrhizobium/Rhizobium from guar nodules and used these strains to inoculate guar. They reported that the inoculated treatments had greater root and shoot elongation, as well as increased nodules per plant and greater plant productivity in comparison to uninoculated treatments. Thapa et al. (2018) observed abundant nodulation in uninoculated guar in contrasting alkaline soils and reported no improvement in nodulation or other plant parameters with application of two inoculants. The authors noted this result may have been due to high populations of indigenous Rhizobia in the local soil. In a field study, Gresta et al. (2019) reported no

active presence of native soil Rhizobia compatible with guar and therefore no nodules in the absence of an inoculant, but there was substantial nodulation when inoculated with CB3035 (BASF, Australia) with an average of 13.8 nodules per plant. In several studies in which only aboveground responses were measured (no nodule measurements), Rhizobium/Bradyrhizobium inoculants were shown to improve plant height, shoot weight, protein content, number of pods, and yield in guar (Elnesairy et al., 2016; Elsheikh and Ibrahim, 1999; Ibrahim et al., 2010; Ibrahim et al., 2016).

Although several studies have tested Rhizobium/Bradyrhizobium strains as inoculants for guar, there is currently almost no commercial availability of inoculants for this crop (Table 1.1.). A guar-specific inoculant, CB3035 from BASF (Australia), is commercially available. CB3035 originated from the CSIRO Tropical Agriculture genetic resource collection and was transferred to industry via the Australian Inoculants Research and Control Services (AIRCS) after field and lab trials (Eagles and Date, 1999). In the United States, inoculants for guar were available in the past, but none are currently commercially available (Trostle, 2017). The companies that developed such products included AGCARES (Dawson County, Texas), Verdesian Life Sciences (Cary, North Carolina), and Sono Ag (Plainview, Texas). Four strains of *Rhizobia* housed in the USDA National Rhizobium Germplasm collection are listed as compatible with guar, but little has been published on their efficacy and none are currently available as commercial inoculants. The University of Hawaii NifTAL (Nitrogen Fixation by Tropical Agricultural Legumes) Center isolated several strains of *Bradyrhizobium*, which were tested on guar in Sudan (Elsheikh and Ibrahim, 1999; Ibrahim et al., 2011;

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Ibrahim et al., 2016), though we were unable to verify if any of the strains had been commercialized. Also in Sudan, local strains of *Bradyrhizobium* were isolated and used for research on guar (Elnesairy et al., 2016; Elsheikh and Ibrahim, 1999; Ibrahim et al., 2011; Ibrahim et al., 2016), but we were also not able to confirm if these have been commercialized. The majority of guar production in the world takes places in India and Pakistan, but little is published on guar inoculants from these countries (Chaudhary and Sindhu, 2016; Khandelwal and Sindhu, 2012; Yadav, 1984) and we could not verify commercial availability any guar inoculant there.

The lack of availability of inoculants for guar is a significant limiting factor in expanding ecosystem services from the crop, including increasing yield and nitrogen fixation. As described earlier, the USDA National Rhizobium Germplasm Collection has strains listed as compatible with guar (USDA 3089, USDA 3385, USDA 3386, and USDA 3595), though little is known about their performance. Our objective was to test the efficacy of four inoculants derived from these strains, as well as a species mixture, on root nodulation, plant nitrogen, and plant productivity in a controlled-environment study. We hypothesized that all five inoculants would increase root nodulation, nitrogen assimilation, and productivity of guar relative to an un-inoculated control.

2.2. Materials and Methods

2.2.1. Experimental Design

This research was fully replicated and repeated in a greenhouse at the Texas A&M AgriLife Research and Extension Center in Vernon, Texas, USA. The

experimental units were 7.6 L black pots. The experimental treatments were five peat-based inoculants, plus an uninoculated control. There were four replicate pots per treatment, giving 24 pots per iteration of the study. These were arranged in a completely randomized design in three rows on greenhouse benches. The first iteration of the study began with guar planting on 3 May 2019 and was terminated 20 June 2019 (48 days); the second iteration was started on 29 June 2019 and terminated on 18 August 2019 (50 days).

2.2.2. Experimental Procedures

A Miles loamy fine sand (fine-loamy, mixed, superactive, thermic Typic Paleustalfs) soil from Locket, Texas was collected from a field that has no direct modern agricultural history and used as the growth medium. Fresh soil was collected for each iteration of the study and the pots were washed thoroughly before beginning the studies to ensure that there was negligible native or inoculant-based Rhizobium contamination. Pots were filled with soil and watered, the soil level was adjusted to within 3 cm of the top, and then watered again before planting. The physical and chemical parameters of the soil used in this study were measured by a commercial lab (Water's Agricultural Lab, Camilla, Georgia, USA) (Table 1.2).

Guar (Lewis variety) seeds were obtained from Texas Foundation Seed Service (Vernon, Texas). Seeds were planted in 7.6-L pots and grown for approximately 50 days. The black pots were wrapped in aluminum foil to prevent radiant heating of the soil above ambient temperatures. Seeds were inoculated immediately before planting with

custom peat-based powder inoculants, which were prepared in a microbiology lab using standard techniques. The six inoculant treatments included: No inoculant (control), USDA 3089, USDA 3385, USDA 3386, USDA 3595, and a mixture of all four USDA accessions. The four Rhizobia strains were chosen for testing from the USDA national Rhizobia collection, because they were listed as compatible with guar and one is known from a preliminary sterile culture experiment (unpublished data) to effectively nodulate and fix nitrogen in guar. To inoculate the seed, 50 g of seed was added to a one-liter sealable plastic bag, 2 g of water was added and mixed to wet the seed, then 3 g of inoculant was added and mixed until the inoculant evenly coated all seeds. This protocol was followed separately for each inoculant, with care taken to avoid cross-contamination among treatments in the lab and greenhouse.

In planting the seeds, six shallow holes were formed and two to three seeds were planted per hole, approximately 2 cm below the soil surface. For the first week following planting, the seeds were watered lightly every day until germination occurred. Plants were thinned to one plant per pot about 10 days after planting. After the first week, watering times and amounts depended on atmospheric conditions and evapotranspiration from the pots; watering times were determined by observation of dry soil in the top 3 cm of the pots and watering amounts were determined by a gravimetric technique. When dry soil conditions were observed, four random pots were weighed and an average water deficit was calculated based on an established set point (full container capacity) that was determined prior to beginning the studies. Water was added back to reach 80% of full

container capacity. The temperature and humidity in the greenhouse were monitored continuously using a Sigma (MODEL) sensor/datalogger module.

Every 10 days, plant height, total nodes on the main stem, and total number of flowering/fruiting nodes were measured. At 50 days after planting, plants were clipped at soil level, placed in paper bags, and dried in an air-forced drying oven at 55 ºC until dry (about 4 days). Once dry, samples were weighed to determine dry biomass. Dried biomass samples were ground using a Wiley Mill to pass through a 2 mm screen and analyzed for total nitrogen content and protein content based on a multiplier of 5.87 (Azero and Andrade, 2002) in an Elementar Vario Max Cube (Elementar Americas, Inc., Ronkonkoma, NY) using the Dumas method (Schindler and Knighton, 1999). The soil in the pots was deconstructed and sifted by hand to remove all root nodules. Nodules were counted, washed with water, then placed in paper bags and dried using the procedure just described. When dry, nodules were weighed to determine total nodule dry weight per pot or per plant.

2.3. Statistical Analysis

Statistical analysis was performed with the SAS 9.4 software (SAS Institute Inc., Cary, North Carolina, USA). The data were analyzed by ANOVA using the GLMMIX procedure. The data for both iterations of the study were analyzed together. Inoculant was considered a fixed effect, while iteration of the study was considered a random effect in the statistical model. The data were checked to ensure they satisfied the assumption of normality and equal variances using histograms, Q-Q Plots, and plots of

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residuals. Degrees of freedom were determined using the Kenward-Roger method.

Treatment differences were determined using statistical contrasts, directly comparing the

treatments individually and collectively to the control for each response variable. All

treatment effects were considered significant at $P < 0.05$.

2.4. Results

2.4.1. Aboveground Plant Parameters

Plant height did not differ in response to the tested inoculants relative to the

uninoculated control (Table 1.3.). The plant height in the control averaged 47.2 cm,

while the inoculant treatment plant averages ranged from 45.3 to 48.7 cm, and the

collective statistical test of all inoculants compared to the control had a P-value of

0.9245. The total number of nodes on the main stem of the plant, as well as fruiting and

flowering nodes, also did not differ in response to the inoculants relative to the

uninoculated control (Table 1.3.). Total nodes in the control averaged 17.0, while the

inoculant treatment plant averages ranged from 17.3 to 18.5, and the collective statistical

test of all inoculants compared to the control had a P-value of 0.2848. Fruiting and

flowering nodes in the control averaged 16.5, while the inoculant treatment plant

averages ranged from 13.6 to 18.8, and the collective P-value was 0.6014. Aboveground

biomass was greater than the control in response to two inoculants, USDA 3089 ($P =$

0.0155) and USDA 3386 ($P = 0.0022$), and the collective statistical test of all inoculants

compared to the control was also significant with a P-value of 0.0197 (Table 1.3.).

Aboveground biomass in the control averaged 18.8 g, while the average biomass in response to the inoculants varied from 19.5 to 23.2 g.

2.4.2. Belowground Plant Parameters

The total number of root nodules per plant did not differ in response to the tested inoculants relative to the uninoculated control (Table 1.4.). The number of nodules in the control averaged 17.1 per plant, while the inoculant treatment averages ranged from 17.1 to 26.1 nodules per plant. The collective statistical test of all inoculants compared to the control had a P-value of 0.3926. Nodule number with the USDA 3385 inoculant was trending toward a significant difference from the control ($P = 0.0669$). Nodule weight was greater than the uninoculated control in response to four inoculants: USDA 3089 ($P = 0.0362$), USDA 3385 ($P = 0.0121$), USDA 3386 ($P = 0.0009$), and the Mix ($P = 0.0433$). The collective statistical test of all inoculants compared to the control was also significant ($P = 0.0062$) (Table 1.4.).

2.4.3. Chemical Composition and Protein Parameters

The chemical composition of the aboveground biomass varied among inoculant treatments, depending on the chemical parameter (Table 1.5.). There was no effect of the inoculants on the percent carbon or nitrogen in the plant tissues, as well as the carbon to nitrogen ratio. Percent protein in plant tissues was directly calculated from the percent nitrogen, thus this also did not differ among inoculant treatments (Table 1.6.). Total assimilated nitrogen was greater than the uninoculated control for USDA 3089 (P =

0.0077) and USDA 3386 (P = 0.0076). Similarly, total protein production was greater for

USDA 3089 (P = 0.0077) and USDA 3386 (P = 0.0076). Soil test results showed that the

soil had low levels of available nitrogen in the forms of nitrate and ammonium (Table

1.2.). The air temperature was somewhat high during the second iteration of the study

than the first. For study iteration 1 and 2, the average daily temperature was 28°C and

31°C, respectively (Figure 1.1.).

2.5. Discussion

Several research groups around the world have isolated *Bradyrhizobia/Rhizobia*

strains compatible with guar (Chaudhary and Sindhu, 2016; Elnesairy et al., 2016;

Elsheikh and Ibrahim, 1999; Ibrahim et al., 2011; Ibrahim et al., 2016; Khandelwal and

Sindhu, 2012). Some of these strains have been tested in controlled experiments and

field trials and, in many cases, have increased guar plant productivity and nodule

formation (Bhardwaj, 1974; Gresta et al., 2019; Khandelwal and Sindhu, 2012; Stafford

and Lewis, 1980; Thapa et al., 2018). Despite this research and development, to our

knowledge only one Rhizobium strain is currently commercially available for guar

(CB3035, Australian Inoculants Research Group). There were commercial inoculant

products available in the past in the United States and perhaps in other regions but are no

longer available. The primary roadblocks to sustainable commercialization of inoculants

for guar are economic factors, as guar is a minor crop in most countries compared to

major legumes like soybean that have profitable commercial inoculant sectors (Abidi et

al., 2015; Trostle, 2017). Limited crop production limits the economy of scale for any

potential guar inoculant and this effect is compounded by the highly species-specific

pairing between compatible Rhizobia species and guar (Abidi et al., 2015), which

prevents any guar inoculant from being marketed for use on other legume crops. Given

these factors, the most likely routes to making inoculant products for guar more widely

available are through the non-profit sector and/or through decreasing inoculant

development costs, through work such as the current research.

In the current study, two of the tested inoculants stood out as most effective:

USDA 3089 and USDA 3386. These had the greatest positive impacts on increasing

nodule weight, total assimilated nitrogen, and ultimately biomass and protein production.

Biological nitrogen fixation (BNF) was not directly measured in this study, but nodule

weight can be used as an indicator of legume fitness and capacity for BNF (Hardarson

and Danso, 1993; Nigam et al., 1985; Pimratch et al., 2008), which has been associated

with improvements in assimilated nitrogen, biomass, and protein in many legumes

(Provorov and Tikhonovich, 2003). Among all five tested inoculants, USDA 3386 had

the highest nodule weight with an average of 1.11 g per plant, a 29% increase over the

0.861 g per plant in the control. Nodule weight was greater than the uninoculated control

in response to three additional inoculants, including USDA 3385, USDA 3089, and the

Mix-species inoculant, with improvements of 22, 17, and 16%, respectively. It is

important to highlight that substantial nodule development from native soil Rhizobia was

present with no inoculant, which would have impacted the magnitude of the inoculant

responses. For example, Khandelwal and Sindhu (2012) reported that nine tested

Bradyrhizobium/Rhizobium isolates increased nodule weight in guar by 388% to 2947%

in sterilized chillum jar assembly conditions at 60 days of plant growth when only minor nodulation was present in the uninoculated control. The results of the present study suggest that the Rhizobia introduced through the tested inoculants, particularly USDA 3089, 3385, 3386, and the mix, were more competitive or effective nodulators than the native soil Rhizobia.

Following strong impacts on nodulation, the USDA 3089 and 3386 inoculants also increased aboveground biomass. USDA 3089 increased biomass by 18% and USDA 3386 increased biomass 23%, relative to the control. No other measured aboveground plant morphological or productivity parameters, including plant height, main-stem nodes, and reproductive nodes, were impacted by the inoculants (Table 1.3.). Others have reported a variety of positive responses to inoculants in guar, the magnitude of which depend on the condition of the control. Ibrahim et al. (2010 and 2011) measured increases in plant height, fruiting branches, and number of pods in response to an inoculant in field experiments on guar at multiple locations in Sudan. Elsheikh and Ibrahim (1999) reported increased seed yield of guar in the field with a Bradyrhizobium inoculant compared to an uninoculanted control. Elnesairy et al. (2016) conducted a field experiment combining inoculation and farmyard manure application and showed that inoculant treatments significantly increased plant height, shoot dry weight, and pod dry weight. In a field experiment, Gresta et al. (2019) reported no difference in plant height or pods per plant with an inoculant, but an increase in seed yield. Ibrahim et al. (2016) reported that a Bradyrhizobium inoculant increased plant height, number of fruiting

branches, number of pods per plant, seed yield, fresh and dry shoot weight, and number of nodules in a field study in Sudan.

Guar has long been used as a multi-purpose legume crop in some areas (Patnayak et al., 1979; Gohl, 1982) and these results further support its use as a forage for livestock due to its protein production. In this study, protein content for all aboveground plant material at 50 days after planting averaged 15.4% (Table 1.6.) in a soil with poor fertility (Table 1.2.). The inoculants did not increase protein content, but they did increase total protein production. Both USDA 3089 and USDA 3386 increased total protein production by 22%. Das et al. (1974) compared crude protein (CP) of 14 guar genotypes in Hissar, India and reported the genotypes averaged 16% CP at first appearance of pods. Singh et al. (1997) evaluated 526 guar germplasm cultivars in Jhansi, India, and reported averages of 17% CP in guar forage. In the Southern Great Plains, Rao and Northup (2009a) reported guar forage biomass to contain16.2–22.5% CP at 45 days after planting. Specifically, for the Southern Great Plains, these values indicate that guar could be used as a high-quality supplemental hay in the late summer, diversifying agricultural production in the region (Rao and Northup, 2013).

Some management aspects and physical dynamics of this controlled-environment study contrast with typical conditions for guar in the field and should be considered in interpretation of the results. High temperature and water stress are major factors affecting the development, function, and viability of root nodules in legumes, including drought-tolerant legumes like guar (Arayanghoon et al., 1990; Sprent, 1976; Stafford and McMichael, 1991; Vekateswarlu et al., 1983). The ambient temperatures recorded in

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this study were similar to those reported outdoors, though soil temperature would be expected to be more homogenous in the pots and to vary more with soil depth in the field. Similarly, the soil moisture regime was more consistently favorable in the pots than would be expected in the field, particularly than in the rainfed or dryland conditions in which guar is most commonly produced (Whistler and Hymowitz, 1979). The perception that guar often does not nodulate effectively in dryland conditions the field (Abidi et al., 2015; Khandelwal & Sindhu, 2012) is likely related to constrains in forming and maintaining nodules in the surface soil layer, which is where most observations of legume nodules are made and where the direct impacts of low soil moisture are most acute. In the pots, the plants never experienced severe water stress and no portion of the root system experienced extreme dryness.

2.6. Conclusions

Guar nodule parameters, nitrogen assimilation, and ultimately biomass and protein production were positively influenced by the inoculants tested in this study, particularly USDA 3386 and USDA 3089. The results of this study illustrate the benefits of inoculating guar to increase the capacity of the crop for BNF, improving its productivity and use as forage. There is only one known inoculant for guar that is currently commercially available, but there are several promising strains of Rhizobia that have been shown to increase plant productivity and nodulation in guar (Bhardwaj, 1974; Gresta et al., 2019; Khandelwal and Sindhu, 2012; Stafford and Lewis, 1980; Thapa et al., 2018), including the tested strains contained in the USDA National Germplasm

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Collection. These strains, particularly USDA 3386 and USDA 3089, should be tested in

the field to further evaluate their effectiveness as an inoculant for guar.

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3. THE ROOT SYSTEM OF GUAR: TEMPORAL ANALYSIS OF PHYSICAL AND STRUCTURAL DEVELOPMENT OF ROOTS AND NODULES

3.1. Introduction

Guar (*Cyamopsis tetragonoloba* L.) is an annual summer legume grown in semi-arid regions around the world, with production centered in India and Pakistan, and to a lesser extent in the United States, South Africa, and Australia (Gresta, et al., 2019). Today it is primarily grown as a grain crop for the galactomannan gum in the seed endosperm, which is used in many industries such as food, paper, textiles, pharmaceuticals, cosmetics, as well as petroleum (Mudgil et al., 2014; Yadav et al., 2015). Guar has been characterized as a drought-tolerant and deep-rooted plant, exceptionally well adapted to semi-arid climates, where water is often the most limiting factor in its production (Alexander et al., 1988; Whistler and Hymowitz, 1979). One mechanism contributing to the tolerance of guar for high temperatures and drought may be that the crop has been shown to access and deplete water deep within the soil profile (Alexander et al., 1988). Deep rooting and maintaining high root densities appear to be major adaptive mechanisms for drought tolerance in legumes generally (Pandey et al., 1984).

There is extensive information available on root physiological traits that enable effective acclimation to heat stress and drought tolerance in legumes (Nadeem et al., 2019; Xiong et al., 2020). In particular, root density, root depth, and nodulation have been widely reported for several legumes such as soybean (Fried et al., 2019; Kulkarni et

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al., 2017; Wu et al., 2007), common bean (Figueriredo et al., 2008; German et al., 2000; Ferreira et al., 2018), peanut (Nigam et al., 1985; Pimratch et al., 2008), chickpea (Chen et al., 2017; Ramamoorthy et al., 2017), lentil (Chanway et al., 1989; Kumar et al., 2012), alfalfa and clovers (Bell, 2005; Goh et al., 2016), but there is little information on other legumes, including guar. There has been significant advancement in characterizing and understanding aboveground shoot growth and development of guar (Adams et al., 2020a; Meftahizadeh et al., 2019; Singla et al., 2016; Stafford et al., 1987), but similar advancement in knowledge is needed on the belowground portion of the plant, especially with regards to the spatial patterns of root growth and the timing of nodule development and senescence. The following paragraphs describe what has been reported in the literature on the root system and nodulation in guar.

One study characterized initial root growth and development in guar seedlings over a 10- day period grown at five different temperature regimes in a lab setting, providing some of the earliest recordings of primary root length, number of laterals, lateral root length, and branching intensity in guar seedlings (Stafford and McMichael, 1990). Another study examined root morphology in guar by examining the role of the root system in salt tolerance and observed a decrease in root growth with a progressive increase in salt stress in pots in a net house in Pakistan (Ashraf et al., 2005). A more recent pot culture study in Pakistan evaluated the root performance of 25 guar genotypes at 15, 30, and 45 days after sowing and results showed a wide variation in length and weight of roots at different salinity levels (Suthar et al., 2018). Other studies have made observations on changes in root morphology of guar under stressful conditions such as

drought and high temperatures (Arayangkcon et al., 1990 Stafford et al., 1991;

Venkateswarlu et al., 1983). Each of these reports provided results on the responses of

the guar root system to various environmental factors, but no studies have been

conducted to show the basic biological development of the root system of guar across

space and time.

More studies have reported on nodulation in guar. In an extension bulletin,

Stafford and Lewis (1980) reported a range of 0.5 to 8.9 nodules per plant in a field

experiment in north Texas and noted that more nodules were found on plants harvested

at 8 weeks after planting than plants harvested at either 4 or 12 weeks. This is the only

scientific report known to provide any information on temporal dynamics of nodule

development in guar at several points over the growing season. An early study by

Bhardwaj (1974) reported an average of 4.63 nodules per plant after 60 days of growth

in a field study with soil amendments such as gypsum and manure as treatment factors.

Brar and Singh (2017) found an average of 8-10 nodules per plant at 50 days when

comparing four guar varieties in a field study in India. Thapa et al. (2018) and Hinson

and Adams (2020a) showed nodules per plant ranging from 0.25 to 43.75 in greenhouse-

grown guar plants at 50 DAP (R2 or R3 stage of guar development), with nodulation

affected by soil and nitrogen treatment factors. Gresta et al. (2019) reported an average

of 13.8 nodules per plant at harvest (approximately 140 days) in guar in a field trial in

Italy where they evaluated the effects of Rhizobium inoculation and phosphate

fertilization on the productive and qualitative traits of guar. Each of these studies

provided snapshots of nodulation in guar in various cultural situations, but no studies

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have rigorously investigated season-long patterns of nodule development and senescence in space and time.

Researchers have reported information on many morphological, biological, and productive traits of guar, but very little is known about root system and nodule development in the plant, which leaves large gaps of knowledge regarding critical belowground functions of this legume. The objective of this study was to investigate temporal and spatial dynamics in root system physical and architectural traits of guar, including dynamics in nodule growth and senescence. We hypothesized that the root system would rapidly grow deep, that nodules would be concentrated toward the soil surface, and that nodules would begin to senesce before harvest maturity.

3.2. Materials and Methods

3.2.1. Experimental Design

This research was fully replicated and repeated in a greenhouse at the Texas A&M AgriLife Research and Extension Center in Vernon, Texas, USA. The experimental units were soil columns, which were arranged in a completely randomized design in two rows in the greenhouse. There were a total of 12 columns per iteration of the study. All 12 columns were managed in the same way, but were randomly assigned to four time-interval or harvest timing treatments, giving three replications of each. At each interval, the assigned columns were destructively harvested, as described in the next section, separating each column into 5 sections by depth.

3.2.2. Experimental Procedures

The first iteration of the study began with guar planting on 20 May 2019 and was terminated 6 August 2019 (78 days); the second iteration was started on 8 May 2020 and terminated on 27 July 2020 (80 days). The columns were PVC pipes that were 130 cm tall and 20.3 cm in diameter. The columns were open at the bottom and root growth and access to water was not restricted to the column. A Miles loamy fine sand (fine-loamy, mixed, superactive, thermic Typic Paleustalfs) soil from near Locket, Texas was collected from a field that has no direct modern agricultural history and loaded into the columns to be the growth medium. The soil was stratified by depth: the top 0 to 30 cm sections of the column came from surface soil (top 30 cm) and the bottom 30 to 125 cm sections came from sub-surface soil (30-60 cm) in the field. Soil was sifted through 2 mm mesh strainers to remove any roots or large pieces of organic matter before filling the columns. After they were filled with soil, sufficient water was added to bring the soil moisture to field and allowed to settle, then surface soil was added to adjust the soil level within 5 cm of the top of the column.

Before planting, seeds were inoculated with a peat-based inoculant that included a mixture of four Rhizobia strains from the USDA germplasm collection: USDA 3089, USDA 3385, USDA 3386, and USDA 3595. These strains are known to effectively nodulate guar (MacMillan et al., 2020). The inoculants were custom preparations, made in a microbiology lab using standard techniques. Inoculant was applied to the seed immediately before planting using the following protocol: 50 g of seed was added to a 1 liter sealable plastic bag, 2 g of water was added and mixed within the bag to evenly coat

the seed, then 3 g of inoculant was added and mixed within the bag until evenly coated on all seed. In planting the seeds, six shallow holes were formed and approximately 2-3 seeds were planted per hole, approximately 2 cm below soil surface. Plants were thinned to one plant per column at about 10 days after planting.

For the first week following planting, the seeds were lightly watered every day until germination occurred. After the first week, watering was done to simulate rainfed conditions, as closely as could be done given the constraints of the system. Watering amounts and times depended on atmospheric conditions and ET, with a target of 90% ET replacement following a drying period. The rate of ET was determined by gravimetrically monitoring the water use of proxy plants. The proxy plants were grown in two 64-liter pots filled with the same soil and planted on the same date as the columns. Preliminary work was done to establish weight set points based on the water holding capacity of the pots. Depending on atmospheric conditions and plant water use, watering typically occurred every 2 to 5 days. The temperature and humidity in the greenhouse were monitored continuously using an Omega OM-92 data logger. Triple super phosphate fertilizer was applied in a liquid suspension to each column in a concentration of 0.3 g of fertilizer per column at 20 DAP in 2019 and 40 DAP in 2020. A syringe was used to apply four 10 ml injections of fertilizer suspension around the edge of the columns. The columns were given 500 mL of water following fertilizer injection to achieve better fertilizer infiltration.

Every 10 days, plants were measured for height, number of nodes on the main stem, and total number of flowering/fruiting nodes on the plant. Columns were

deconstructed at regular intervals: 20, 40, 60, and 80 DAP. During deconstruction, the aboveground portion of the plants were clipped at soil level, placed in paper bags, and dried in an air-forced drying oven at 55ºC until dry or about 4 days. Once dry, samples were weighed to determine biomass. The soil in the columns was separated into five sections (0-15 cm, 15-30 cm, 30-60 cm, 60-90 cm, and 90-125 cm) and sifted by hand to remove all living roots and nodules. The roots were analyzed for physical and morphological parameters using WinRHIZO (Regent Instruments Inc., Canada), then dried to determine root mass. Nodules were counted to determine nodule number, then washed, dried, and weighed to determine total nodule dry weight per column or per plant. Rooting depth was recorded at each harvest interval until to the depth exceeding the length of the columns. Each time a column was deconstructed, a portion of the nodules were checked at random for internal color, with red signifying healthy, active nodules, and grey/green signifying senescing nodules.

3.3. Statistical Analysis

The objective of this study was to provide information on whole-plant development in guar and, to better highlight differences between the iterations of the study, the data for each year were analyzed and presented separately. Means and standard errors are presented for both above- and belowground plant parameter measurements over space and time. In cases where comparisons are made, means were considered different when their error terms did not overlap. Using the PROC REG procedure in the SAS 9.4 software (SAS Institute Inc., Cary, North Carolina, USA),

linear and quadratic regression models were tested for their fit to the data for total above-
and belowground plant variables over time (Table 2.6.). A probability threshold of 0.05
was used to determine statistical significance for the regression models. In the case that
both linear and quadratic relationships were significant for a particular variable, the more
significant one was presented.

3.4. Results

3.4.1. Soil and Weather Data

Surface and subsurface soils were mixed for nutrient analysis for both years of
the study and results are given in Table 2.1. Levels of soil NO_3-N were higher in 2019
(10.0 mg kg^{-1}) than 2020 (5.5 mg kg^{-1}), as were the levels of soil NH_4-N (2.05 mg kg^{-1} in
2019 and 0.38 mg kg^{-1} in 2020). The levels of most micronutrients detected in the soil
were higher in 2019 than in 2020 (Table 2.1). The pH of the soil was comparable across
years of the study, with 2020 having a slightly higher pH (7.5) than 2019 (7.2). The soil
in 2019 had higher organic matter (0.68%) than was detected in 2020 (0.21%).

3.4.2. Aboveground Parameters

In both 2019 and 2020, plant height, number of main stem nodes, and the total
number of fruiting and flowering nodes per plant had a quadratic relationship over time,
while biomass had a linear relationship (Table 2.6). The measured values of all
aboveground plant parameters (Table 2.2) were greater in 2019 than in 2020, with the
exception of plant height, which was similar between years. Plant height at harvest (80

DAP) averaged 97.3 cm in 2019 and 93.7 cm in 2020. In 2019, the total number of nodes on the main stem increased at a steady rate until harvest when the average was 37 nodes per plant. In 2020, the number of nodes on the main stem plateaued around 60 DAP and had an average of 25 nodes per plant at harvest. Flowers were first observed by 40 DAP in 2019, followed by exponential development of fruits and flowers until harvest (80 DAP), averaging 130 fruiting and flowering nodes per plant. In 2020, flowers were first observed by 30 DAP and grew at a steady rate until harvest with an average of 54 fruiting and flowering nodes per plant, substantially less than the previous year. Aboveground biomass increased at similar rates both years but was higher at harvest in 2019 (167 g) than 2020 (135 g) (Table 2.2).

3.4.3. Belowground Parameters

In both years of the study, a portion of nodules showed a grey/green color beginning at 60 DAP. In 2019, nodule number was substantially greater at the soil surface (0-15 cm depth) than at deeper soil layers early in the growing season, peaking at 40 DAP (Figure 2.1). Nodule number continued to increase at deeper soil depths until at least 60 DAP. By 60 DAP, nodule numbers at 0-15 cm and 15-30 cm were similar. By 80 DAP, the timing that final observations were made, nodule numbers decreased with soil depth as a general trend. Drastically fewer nodules were formed at all soil depths in 2020 than in 2019, with no consistent trends over time apparent in nodule numbers with depth. In both years, nodule number showed a positive linear relationship over time (Table 2.6).

In 2019, nodule weight was minimal at all soil depths at 20 DAP but had increased by 40 DAP in the surface soil layer (0-15 cm) (Figure 2.1). In 2020, nodule weight was minimal at 40 DAP, but had increased by 60 DAP. Once nodule weight started to increase in the surface soil, it increased until 80 DAP, with no slowing of the rate of growth evident in the data. Similar to nodule number, increases in nodule weight in deeper soil layers were delayed relative to nodule weight at the soil surface in 2019, but also increased over time. Nodule weight was negligible from 60-125 cm soil depth over the 80-day course of measurement in both years. In 2019, the average weight per nodule with depth was 0.060 g at 0-15 cm, 0.050 g at 15-30 cm, 0.028 g at 30-60 cm, 0.010 g at 60-90 cm, and 0.0085 g at 90-125 cm. In 2020, the average weight per nodule with depth was 0.095 g at 0-15 cm, 0.076 g at 15-30 cm, 0.052 g at 30-60 cm, 0.038 g at 60-90 cm, and 0.026 g at 90-125 cm. Like nodule numbers, there was less nodule weight at all soil depths in 2020 than in 2019, with no consistent trends apparent over time in nodule weight with depth in 2020 (Table 2.3). In 2019, nodule weight showed a quadratic relationship with time, while in 2020 nodule weight showed a more linear trend (Table 2.6). Averages for nodule parameters at every harvest interval (20 days) are available in Table 2.3.

Among all soil layers, root length density (RLD) was the greatest at 0-15 and 15-30 cm soil depths for all time intervals (Figure 2.1). The RLD values and trends over time were similar between these layers in both years. There was minimal RLD from 30-125 cm soil depth over the 80-day course of measurement in both years. Average RLD

for the whole column was greater in 2020 than in 2019 (Table 2.4). In both years, the overall trend in RLD over time was positive and linear (Table 2.6).

The trends in specific root length (SRL) were similar by soil depth in both years of the study. The smallest SRL values were observed at the soil surface, increased until the 30-60 cm depth, and then decreased again in deeper soil layers. SRL ranged from 13 – 48 m g^{-1} in 2019 and 19 – 60 m g^{-1} in 2020 (Table 2.5). Analysis of root diameter classes with soil depth showed that the greatest percentage of root length was comprised of roots less than 0.5 mm in diameter at all soil depths (Table 2.5). In both years, 86% or more of all root length was comprised of roots less than 1 mm in diameter at all soil depths, with a strong skew toward the finer roots less than 0.5 mm in diameter. At the soil surface, there was roughly 5 to 7% of root length in the 1 to 2 mm diameter class. The percentage of root length in the 1 to 2 mm class was generally less than 2% below the surface layer in 2019, though greater percentages were recorded in 2020. There was generally less than 1% of root length comprised of roots greater than 2 mm at all soil depths.

In 2019, root weight was highest in the surface layer at all time intervals, peaking at 80 DAP (Figure 2.1). In 2020, root weight was minimal at all depths until 20 DAP when it began to increase in the surface layer with no slowing of the rate of growth evident in the data. In 2019, root weight was minimal in the 60-125 cm soil depth, while in 2020 root weight was minimal in the 30-125 cm soil depth over the 80-day course of measurements. In contrast to aboveground biomass, average root weight for the entire column was greater in 2019 than in 2020 (Table 2.4). In both years, the root weight had

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a positive quadratic trend over time (Table 2.6). In both years of the study, the root to shoot ratio showed the same general trend over time with the smallest ratio at 20 DAP and the greatest ratio at 40 DAP. The root to shoot ratio ranged from 0.063 to 0.12 in 2019 and 0.055 to 0.17 in 2020 (Table 2.4). Averages for root parameters at every harvest interval (20 days) are available in Table 2.4.

3.5. Discussion

There was a noticeable difference in nodulation and aboveground productivity between years of the study. In 2019, the average number of nodules and nodule weight per plant were higher at each harvest interval than in 2020, coinciding with higher aboveground biomass at each harvest interval as well. In contrast, growth and development of the root system was similar between years in both space and time. Given the similarity in the root system, which can have a substantial impact on aboveground plant performance (Noble and Rogers, 1994), it seems reasonable that decreased nodulation and subsequent nitrogen limitation in 2020 may have been a primary cause for the lower aboveground biomass productivity in that year.

Typically, the N-fixation period in legumes is optimal between 4 and 5 weeks after Rhizobia infection and then N-fixing bacteroid capacity is reduced, nitrogenase activity declines, and senescence begins (Dupont et al., 2012). As N-fixing activity decreases over time, the red N-fixing tissues within nodules become grey/green in color due to the breakdown of leghemoglobin (Lehtovaara and Perttila, 1978). This process occurs naturally over time, or can be induced by exogenous factors, such as high soil

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nitrate levels, or stress such as salt stress, dark stress, or drought stress (Dupont et al.,
2012; Matamoros et al., 1999). In both iterations of this study, green/grey nodule
interiors were first observed at 60 DAP, signifying the beginning of senescence and
inactive nodules around this time. Increases in nodule weight were documented between
60 and 80 DAP, however, suggesting some continued BNF activity during that
timeframe.

Nodule weight has been shown to be more indicative of legume fitness and
capacity for BNF than nodule number (Hardarson et al., 1993; Nigam et al., 1985;
Pimratch et al., 2008). A novel aspect of the current study is that nodule number and
weight information are shown with soil depth in guar for the first time, giving insight
into the belowground spatial dynamics of BNF. The data shows that nodules were
concentrated toward the soil surface and that the average weight per nodule also
decreased with soil depth. In both years, there was a noticeable trend of nodule size
decreasing with depth. The small numbers and size of the nodules at deeper soil depths
suggests the contribution of these nodules to overall plant BNF may be relatively small.

The concentration of nodules and nodule weight with soil depth would be
expected to affect the function of these nodules and the overall plant capacity for BNF in
the field. Guar is grown in semi-arid regions of the world, most commonly in dryland or
rainfed conditions, where surface temperatures are typically high and soil drying occurs
between rain events, especially at the soil surface (Alexander et al., 1988; Gresta et al.,
2019; Whistler and Hymowitz, 1979). Water and temperature stresses have been
reported to suppress nodule formation and function in guar and other legumes,

consequently causing a decrease in nitrogen fixation (Arayangkoon et al., 1990; Silvente et al., 2012; Venkateswarlu et al., 1983). Given that the bulk of nodule weight was concentrated in the top 15 cm of the soil in the current study, a large fraction of nodules on a field-grown guar plant may be susceptible to loss of function due to dehydration and/or heat stress or may be prevented from forming at all. In this situation, the BNF capacity of the plant may depend on nodules deeper in the soil profile. This may also be the reason that some have speculated that nodulation in guar may be poor in field conditions, following simple assessment of nodulation just at the soil surface (Abidi et al., 2015; Khandelwal and Sindhu, 2012).

The drought tolerance of guar may be due, in part, to the ability of the crop to deplete water deep within the soil profile. Roots reached the bottom of the 130 cm soil columns by 40 DAP both years this study was conducted. Root weight was greatest at the surface (0-15 cm) and decreased deeper in the soil profile, though root length density in the top two layers were similar. The difference in root weight but similarity in root length between the top two soil layers suggests a difference in the coarseness or fineness of rooting between these layers, a pattern consistent in both years and supported by the root diameter class data. In both years, root length density and root weight were minimal in deeper soil layers, with the exception of roots in the 30-60 cm soil layer which increased slightly between 60 and 80 DAP in 2019. Though plants typically have decreasing root density with depth, relatively small amounts of rooting can have an outsized impact when water is limited, enabling the plant to survive or even continue to grow during drought periods (Pandey et al., 1984).

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The root system of guar was dominated by fine rooting from the top to bottom. Using the traditional definitions of fine (< 2 mm) and coarse (> 2 mm) roots (McCormack et al., 2015; Zhang and Wang, 2015), coarse roots generally comprised less than 1% of root length at all soil depths measured. In both years, 86% or more of durable and collectable root length was comprised of roots less than 1 mm in diameter at all soil depths, though the vast majority of this root length was very fine roots less than 0.5 mm in diameter. Most of the roots with diameters greater than 1 mm were found near the soil surface, including about 5 to 7% of total root length within the 1 to 2 mm diameter class and about 1.5% was greater than 2 mm. These relatively heavy roots would have comprised most of the primary root structure of the plant, including the central tap root and larger lateral roots, though the presence of low levels of roots in these diameter classes at all measured soil depths indicates the root system has a hierarchical structure throughout (Table 2.5). Still, it seems that extensive proliferation of fine roots is responsible for the vast majority of root system function in guar.

3.6. Conclusions

The results of this study illustrate the temporal and spatial dynamics in root system physical and architectural traits of guar, including dynamics in nodule growth and senescence. As hypothesized, the root system grew deep rapidly, reaching the bottom of the 130 cm soil column between 20 and 40 DAP. Root weight and root length density generally decreased with depth and the highest percentage of roots were less than 1 mm in diameter at all soil depths. Nodules were present in all soil depths from 40 DAP

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until harvest at 80 DAP, but they began to senesce around 60 DAP. The data shows that

nodules were concentrated toward the soil surface and that the average nodule weight

also decreased with soil depth. Guar is grown in semi-arid regions where surface

temperatures are typically high and soil drying occurs between rain events. Since the

bulk of the nodules and nodule mass were located in the top layers of the soil, these

nodules may be susceptible to loss of function due to dehydration and/or heat stress,

thereby reducing the capacity of guar for BNF in field settings. Further study is needed

to more thoroughly investigate this issue directly in field conditions.

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4. QUANTIFYING BIOLOGICAL NITROGEN FIXATION OF COOL-SEASON LEGUMES IN THREE SYSTEMS IN THE U.S. SOUTHERN GREAT PLAINS

4.1. Introduction

Cool-season legumes, grown as cash and cover crops, are a valuable component of cropping systems. Legumes can reduce nitrate leaching, contribute to long-term accumulation of soil organic matter, suppress weeds, produce high protein foods and feeds, among other ecosystem services (Blesh, 2017; Drinkwater et al., 1998; Finney et al., 2017; Tonitto et al., 2006). Uniquely, legumes are also able to fix N through a symbiotic relationship with root-associated N-fixing bacteria, collectively termed Rhizobia, through a process referred to as biological nitrogen fixation or BNF (McCauley, 2011). Nitrogen deposition from legume-based BNF is essential in organic cropping systems (Connor, 2018), but BNF is gaining more attention as an alternative option for N input in conventional agriculture (Blesh and Drinkwater, 2013; Drinkwater et al., 1998; Ross et al., 2008). Since N is the nutrient that most frequently limits crop production, BNF is one of the most important ecosystem services rendered by legumes in cropping systems and perhaps the largest motivation for producers to grow them (Hungria and Vargas, 2000). In the semi-arid Southern Great Plains region of the U.S., hairy vetch (*Vicia villosa*), Austrian winter pea (*Pisum sativum subsp. arvense*), lentil (*Lens culinaris*), and other species are among the cool-season legumes grown by producers (Guretzky et al., 2012; Sheaffer and Evers, 2007), which are the focus of this research.

Legumes can be incorporated into cropping systems as an alternative way to minimize the use of synthetic fertilizers, with economic and ecological benefits (Ram and Meena, 2014; Yadav et al., 2000). Many studies have shown improvements in the productivity of non-legume crops when they follow legumes in cropping systems (Banyong et al., 2000; Bonilla et al., 2017; Dalal et al., 1998; Meena et al., 2015 Yusuf et al., 2009). Not only can legumes be valuable cover crops in rotation with cotton or cereal crops, they can also be grown as cash crops. Austrian winter pea, hairy vetch, and other cool-season annual legumes are well adapted to the Southern Great Plains and are commonly used as forage in pasture-based livestock production systems or as hay crops (Guretzky et al., 2012; Sheaffer and Evers, 2007).

The rate of BNF varies across cool-season legume species and depends on environmental and plant health factors (Blesh, 2017). Data on this topic is typically reported as percent of N derived from the atmosphere (%Ndfa) and total fixed N (kg N ha^{-1}). Unkovich et al. (2010) reviewed and summarized legume N fixation reports for crops and pastures in Australia from published and unpublished reports. They reported %Ndfa for Austrian winter pea, clovers, vetch, and lentil as 66, 57, 80, and 60%, respectively, and total fixed N as 84, 69, 98, and 61 kg N ha^{-1}, respectively. In a review to update long-standing estimates of biological N_2 fixation for different agricultural systems across the globe, Herridge et al. (2008) reported averages of 63 %Ndfa and 40 kg N ha^{-1} for both lentil and pea. Similarly, in their review of literature from varying environments around the world, Kakraliya et al. (2018) reported N fixation in lentil to range from 40-68 kg N ha^{-1} and Austrian winter pea to range slightly higher with rates

between 65-100 kg N ha^{-1}. Furthermore, van Kessel and Hartley (2000) reported long-term trends in field studies since 1987, showing BNF for pea and lentil averaging 66 and 57 %Ndfa, respectively.

No reports on cool-season legume BNF were found from the Southern Great Plains region. The objective of this study was to quantify BNF and productivity in four cool-season legume species—hairy vetch, Austrian winter pea, crimson clover, and lentil—which are either commonly grown in the region or are of interest to producers, in three distinct management systems. We hypothesized that the percent and total aboveground legume N derived from BNF would vary among legume species and management systems. The information this study provides is critical in understanding the role legumes can play in sustainable agriculture in the region and in promoting the production of legumes to producers as cash and cover crops.

4.2. Materials and Methods

4.2.1. Study Site Descriptions and Experimental Designs

Three distinct study sites were involved in this analysis. All locations are experimental dryland cropping systems in the Texas Rolling Plains subregion of the greater Southern Great Plains region. Site 1 and Site 2 are located in close proximity (~1 km) at the Texas A&M AgriLife Chillicothe Research Station near Chillicothe, TX (34°11′ N, 99°31′ W, 443 m above sea level). The soil at Site 1 is classified as a Grandfield fine sandy loam (fine-loamy, mixed, superactive, thermic Typic Haplustalfs) with 0-1% slope. The soil at Site 2 is classified as an Abilene clay loam (fine, mixed,

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superactive, thermic Pachic Argiustolls) with 0-1% slope. Site 3 is located 16 km south

of Vernon, TX (34°15′ N, 99°27′ W, 361 m above sea level). The soil is classified as a

Wichita Clay loam (fine, mixed, superactive, thermic Typic Paleustalfs) with 1-3 %

slope. All sites are maintained by the Texas A&M AgriLife Research and Extension

Center at Vernon.

At Site 1, a study was initiated in 2011 to evaluate no-till dryland cotton systems

with cool-season cover crops, including Austrian winter pea, hairy vetch, crimson

clover, winter rye, and mixed cover with a comparison to conventional tilled and no-till,

no-cover crop treatments. The system treatments were arranged in a randomized

complete block design with four replications. Cotton was planted as the summer cash

crop each year and no fertilizer has been applied since the onset of the study. Full

experimental and management details for this study are available in DeLaune and

Mubvumba (2020).

At Site 2, a study was initiated in 2018 to evaluate no-till dryland cotton systems

with different seeding rates of cool-season cover crops, including Australian winter pea,

hairy vetch, lentil, and rye. Treatments were arranged in a randomized complete block

design with four replications. Cotton was planted as the summer cash crop each year and

fertilizer was applied before the cotton crop in May 2019.

Site 3 is a study that directly compares conventional and transitional organic

dual-purpose winter wheat cropping systems. The study was established in 2018, with

conventional and organic systems arranged in a randomized complete block design with

four replications per treatment. The transitional organic cropping system consists of crop

rotation (dual-purpose wheat and legume-based hay crops) in the winter and legume-based cover crops in the summer.

4.2.2. Experimental Procedures

Site 1 was seeded with the following cover crops and rates: Austrian winter pea at 33.6 kg ha^{-1}, crimson clover at 16.8 kg ha^{-1}, hairy vetch at 16.8 kg ha^{-1}, and rye at 33.6 kg ha^{-1}. These plots were planted in November 2019 and terminated in April 2020. All legume seed was pre-inoculated (MBS Seeds, Denton. TX). Before harvesting the crops in April, whole plants were dug up and nodules were observed on the roots of all legume species, except crimson clover. Soil sampling for the current report only occurred in the no-till, no-cover crop plots as a representative site reference.

Site 2 was seeded with four different seeding rates per species following cotton harvest. Austrian winter pea was seeded at 16.8, 33.6, 67.3, and 100.9 kg ha^{-1}, hairy vetch was seeded at 5.6, 11.2, 22.4, and 33.6 kg ha^{-1}, lentil was seeded at 8.4, 16.8, 33.6, and 67.3 kg ha^{-1}, and rye was seeded at 16.8, 33.6, 67.3, and 100.9 kg ha^{-1}. This site was planted in November 2019 following cotton harvest and terminated in April 2020. All legume seed was pre-inoculated (MBS Seeds, Denton. TX). Before harvesting the crops in April, whole plants were dug up and nodules were observed on the roots of all legume species. Soil sampling for the current report only occurred in the no-till, no-cover crop plots as a representative site reference.

The hay crop at Site 3 was seeded with a mix of 44.8 kg ha^{-1} of Austrian winter pea, 5.6 kg ha^{-1} common vetch, and 22.4 kg ha^{-1} of winter wheat. Each plot was

approximately 5 ha in area and was planted at the end of September 2019 and harvested at the beginning of May 2020. Legumes were inoculated with Exceed (peat) Superior Legume Inoculant (Vision Biologies, Wichita Falls, TX). In April, whole plants were dug up and nodules were observed on rooting systems. Composted cattle manure was applied at a rate of 4483.4 kg/hectare at Site 3 in Fall of 2018. Sampling from this site was limited to the legume-based winter hay crop consisting of, Austrian winter pea and common vetch, in the transitional organic system in the 2019/2020 winter season, which was in the second year of organic transition.

Weather data was obtained from two weather stations, one at the Chillicothe Research Station that represents Sites 1 and 2, and the other located at Site 3. Twenty years of weather data from the Vernon region was compiled and average temperature and precipitation were calculated for each month in the winter growing season from September to April (Figure 3.1).

Aboveground biomass was collected from all plots at the three experimental locations in April 2020. In the case of Sites 1 and 2, samples were collected from representative spots within each plot at least 0.25 m from the plot edge. In the case of Site 3, four subsamples were collected from representative spots across each plot, combined into a single sample, then partitioned into legume and non-legume components. Negligible amounts of common vetch were present in the hay mixture at Site 3 and so the results from this site refer only to pea. A 2500 cm^2 frame (50 cm x 50 cm) was used to guide the sampling for the legumes (except crimson clover) and mixed-species crops, while a 1-m pole was used to indicate sampling length along the row for

rye and crimson clover. Plant tissue was clipped at 1 cm above soil level, placed in paper

bags, and dried in an air-forced drying oven at 55ºC until dry or about 4 days. Each

sample was weighed and then ground, first in a Wiley Mill to pass through a 2 mm

screen and then in a Cyclone Mill to pass through a 1 mm screen. Each sample was then

processed for $\delta^{15}N$ and $\delta^{13}C$ isotope analysis at the Texas A&M Stable Isotopes for

Biosphere Science Laboratory.

The $\delta^{15}N$ values that resulted from isotope analysis for each plant sample were

entered into the following equation for estimating BNF by the Natural Abundance

Method (Shearer and Kohl, 1986) to achieve the percent of N derived from the

atmosphere (%Ndfa).

$$\%\text{Ndfa} \ = \ \frac{\delta15N \text{ of reference plant} - \delta15N \text{ of N2 fixing legume}}{\delta15N \text{ of reference plant} - B} \ \times \ \frac{100}{1}$$

The B-value is the $\delta^{15}N$ content of the legume when grown with complete

dependence on biological N_2 fixation. This method allows N-fixation to be assessed in

almost any situation where both N-fixing and non-N-fixing plants are present at the same

location. The non-N-fixing plants are reference plants and provide a measure of plant-

available soil N. During BNF, the heavier stable isotope, $\delta^{15}N$, is discriminated against

by bacteria and its subsequent transfer to the plant is less than $\delta^{14}N$ (West et al., 2005).

Therefore, non-N-fixing plants typically have greater $\delta^{15}N$ values than N-fixing plants.

At Site 3, intercropped wheat was used as the reference plant for the Austrian winter pea

mixed with it. At Sites 1 and 2, monoculture rye planted in adjacent plots within

experimental blocks was used as the reference plant. B-values for each legume in this

study were taken from literature and were as follows: -0.56 (Lentil), -0.66 (Austrian winter pea), -0.79 (Hairy vetch), and -1.21 (Crimson clover) (Unkovich et al., 2008).

4.3. Statistical Analysis

Because of the distinct experimental backgrounds and designs, the data for each site was analyzed separately. Statistical analysis was performed for Sites 1 and 2 with the SAS 9.4 software (SAS Institute Inc., Cary, North Carolina, USA), they were analyzed by ANOVA using the GLMMIX procedure. Preliminary statistical analysis of cover crop biomass for Site 2 was performed with seeding rate and species as fixed effects and block was a random effect in the statistical model. Cover crop biomass did not differ among seeding rates; therefore, data was re-analyzed without seeding rate as an effect in the model. For Site 1 species was considered a fixed effect and block was considered a random effect in the statistical model. All data were checked to ensure they satisfied the assumption of normality and equal variances using histograms, Q-Q Plots, and plots of residuals. Degrees of freedom were determined using the Kenward-Roger method. Treatment differences were determined using the Fisher method. All treatment effects were considered significant at $P < 0.05$. For Site 3, means and standard deviation were calculated across replicate plots and presented.

4.4. Results

4.4.1. Soil and Weather Data

Soil nutrient results for all sites are given in Table 3.4. Though they cannot be statistically compared, the highest levels of soil NO_3-N were detected at Site 2 (17.3 mg kg^{-1}), followed by Site 1 (8.53 mg kg^{-1}), then Site 3 (0.790 mg kg^{-1}). Levels of soil NH_4-N were more similar across sites, with 5.78 mg kg^{-1} detected at Site 3, 2.37 mg kg^{-1} at Site 2, and 0.940 mg kg^{-1} at Site 1. The total available N (NO_3-N + NH_4-N) was 19.8 mg kg^{-1} at Site 2, 9.47 mg kg^{-1} at Site 1, and 6.57 mg kg^{-1} at Site 3. Site 3 had the highest soil pH (8.2), with lower and similar soil pH values detected at Site 2 (6.9) and Site 1 (6.6). Weather data for Sites 1 and 2, Site 3, as well as 20-year averages for the region, are given in Figure 3.1. All three study sites had above-average air temperatures in September, December, January, and March and below-average temperatures the remainder of the months when compared to the 20-year average temperatures for the region, though monthly average temperatures never varied substantially from the long-term average. All three sites had above-average precipitation in September and March, but Site 3 also had above-average precipitation in November and January. All sites had below-average precipitation in October, December, and April. The season-long precipitation was highest at Site 3 (403 mm) with a lower value measured at Sites 1 and 2 (340 mm), which was similar to the 20-year average seasonal precipitation (350 mm).

4.4.2. Site 1

Nearly all response variables differed by species at Site 1. Hairy vetch and

Austrian winter pea had the highest biomass (2.82 Mg ha^{-1} and 2.73 Mg ha^{-1},

respectively), then rye (1.95 Mg ha^{-1}), and lastly crimson clover (0.497 Mg ha^{-1}). The

collective statistical test for differences in biomass production among species had a *P*-

value of 0.0001 (Table 3.1). Vetch and pea had the highest N content (3.30% and 3.29%,

respectively), then clover (2.29%), and lastly rye (0.980%). The collective statistical test

for differences in N content among legume species had a *P*-value of < 0.0001 (Table

3.1). Pea had the highest %Ndfa (72.6%) and differed from vetch (54.7%) and clover

(52.6%), which did not differ from each other. The collective statistical test for

differences in %Ndfa for all species had a *P*-value of 0.0532 (Table 3.1). Vetch and pea

had the highest total assimilated N (92.8 kg ha^{-1} and 90.2 kg ha^{-1}, respectively), with

much lower N assimilation values observed in clover (11.7 kg ha^{-1}) and rye (19.3 kg ha^{-1}

^{1}), which did not differ from each other. The collective statistical test for differences in

total N among species had a *P*-value of < 0.0001 (Table 3.1). Finally, pea and vetch had

the highest total rates of BNF (66.7 kg ha^{-1} and 51.3 kg ha^{-1}, respectively), both

substantially greater than clover (6.08 kg ha^{-1}). The collective statistical test for

differences in BNF for all species was had a *P*-value of 0.0012 (Table 3.1).

4.4.3. Site 2

All response variables differed by species at Site 2. Rye had the highest biomass

production (5.09 Mg ha^{-1}), followed by hairy vetch and pea (3.56 Mg ha^{-1} and 3.44 Mg

ha^{-1}, respectively), then lentil (2.54 Mg ha^{-1}). The collective statistical test for differences in biomass for all species had a *P*-value of < 0.0001 (Table 3.2). Pea and lentil had the highest N content (3.72% and 3.63%, respectively), followed by vetch (3.02%), then rye (1.44%). The collective statistical test for differences in N content for all species had a *P*-value of < 0.0001 (Table 3.1). Pea and lentil had the highest %Ndfa (57.2% and 51.8%, respectively), with a substantially lower percentage observed in vetch (34.36%). The collective statistical test for differences in %Ndfa among species had a *P*-value of 0.0048 (Table 3.2). Pea and vetch had the highest total aboveground assimilated N (128 kg N ha^{-1} and 109 kg N ha^{-1}, respectively), though vetch did not statistically differ from lentil (91.8 kg N ha^{-1}); rye had the lowest assimilated N (70.9 kg N ha^{-1}), though it also did not differ from lentil. The collective statistical test for differences in total N for all species had a *P*-value of 0.0056 (Table 3.2). Finally, pea had the highest total BNF (73.9 kg ha^{-1}), with lower values observed in lentil (48.6 kg ha^{-1}) and vetch (45.8 kg ha^{-1}). The collective statistical test for differences in total BNF among species had a *P*-value of 0.0321 (Table 3.2).

4.4.4. Site 3

This site differed from the others, being an intercrop of winter pea, common vetch, and wheat in a transitional organic system. In the intercrop, legume biomass was 1.79 Mg ha^{-1} (dominated by winter pea) and wheat biomass was 1.22 Mg ha^{-1}, giving total biomass of 3.01 Mg ha^{-1} (Table 3.3). The N content of the legumes was 3.21% and for wheat it was 1.18%, giving a weighted total of 2.46% overall (Table 3.3). The

mean %Ndfa for the legumes was 92.7%, with a weighted total BNF (including the non-N-fixing wheat) of 54.6 kg BNF ha^{-1} (Table 3.3). The total assimilated N for the legumes was 58.6 kg N ha^{-1} and for wheat was 14.7 kg N ha^{-1}, giving a sum total assimilated N for the entire crop of 72.6 kg N ha^{-1} (Table 3.3).

4.5. Discussion

4.5.1. Species Comparisons

In monoculture settings (Sites 1 and 2), hairy vetch and Austrian winter pea had the highest biomass production among the legumes tested. Biomass production for pea ranged from 2.7 to 3.4 Mg ha^{-1} and vetch ranged from 2.8 to 36 Mg ha^{-1}. Lentil, which was only grown at one site, produced slightly less biomass (2.5 Mg ha^{-1}), though clover, which was also only grown at one site, produced substantially less than all other legumes (0.50 Mg ha^{-1}). These ranges are similar to average production values reported from another study in the Southern Great Plains of 3.2 Mg ha^{-1} for pea, 3.5 Mg ha^{-1} for hairy vetch, and 1.1 Mg ha^{-1} for clover across four sites in the region (Guretzky et al., 2012). These ranges are also comparable to cover crop biomass production from Site 1 from 2013-2017, where the averages were 2.5 Mg ha^{-1} for pea, 2.3 Mg ha^{-1} for vetch, and 0.75 Mg ha^{-1} for clover (DeLaune and Mubvumba, 2020).

The similarity of the legume biomass productivities for the current study to those reported in the literature from the region are expected, given the similarity of weather parameters in the 2019/2020 winter growing season to 20-year averages for the region (Figure 3.1). Average monthly temperature did not substantially vary from the 20-year

average at any site over the course of the season. Season-long precipitation was just 10

mm less than average at Sites 1 and 2 and Site 3 was 53 mm above average. There were

some notable monthly differences in precipitation that merit discussion. There were high

levels of precipitation in September at all sites, which would have elevated stored soil

moisture for robust early growth. The September precipitation would have been

particularly important in restoring soil moisture at Sites 1 and 2, where summer cotton

crops were nearing maturity at that time. During the period when legumes were actually

growing at Sites 1 and 2, precipitation was above average only in March, though this is a

critical period when growth and N-fixation are maximized in most cool-season legumes

(Jensen, 1987; Unkovich et al., 2010). At Site 3, there was below-average precipitation

in October and December, but above-average levels of precipitation in November,

January, and March that would have supported season-long growth and N fixation.

Although the results show that the biomass productivities of pea and vetch were

similar to each other in this average weather year, the results showed that the two species

could be distinguished as N-fixers by their N content and %Ndfa values. The average N

content in biomass for pea was 3.41% and vetch was 3.18%; the %Ndfa of vetch ranged

from 34.7 to 54.4%, while the rate for pea was higher, ranging from 57.2 to 72.6%.

These values are similar to %Ndfa values for these legumes reported in the literature,

although slightly below average for vetch (Herridge et al., 2008; van Kessel and Hartley,

2000; Unkovich et al., 2010). BNF, which is the product of biomass production, total N

content, and %Ndfa, was therefore greater in pea (66.7 to 73.9 kg ha^{-1}) than vetch (45.8

to 51.3 kg ha^{-1}), depending on the site, and greater than all other legumes tested at both

sites. Reports on lentil from the Southern Great Plains region were not found in the scientific literature, but the performance of lentil at Site 2, including relatively moderate biomass production and high %Ndfa (51.8%), made the rate of BNF in lentil comparable to vetch. The relatively high %Ndfa of clover (52.6%) could not compensate for its poor growth in the region, giving a low rate of BNF.

4.5.2. System Comparisons

Legume N fixation tends to vary with seasonal weather conditions, soil fertility, and management history (Blesh, 2017; Unkovich, 2010). Environmental and management factors, including drought and heat stress, directly impact BNF and its associated functions (Hungria and Vargas, 2000). In this set of studies, there were three sites with varying environmental and management factors. Sites 1 and 2 were dryland cotton systems with a variety of monoculture cool-season legume and rye cover crops and Site 3 was a dual-purpose wheat system in its second year of transition to organic production, with wheat and legumes intercropped for hay in the winter cropping season. In addition to differences in management, soil analysis showed that the sites varied in soil fertility (Table 3.4).

Inorganic forms of soil N nourish legumes, promoting growth, but they (primarily NO_3) also inhibit nodule formation and function (Hinson and Adams, 2020; Streeter and Wong, 1988). Thus, as the availability of soil N increases, rates of %Ndfa and BNF decrease (Chien et al., 1993; Soliman et al., 1995). This may partially explain why the measured rates of %Ndfa were lower at Site 2 than at Site 1 for monocultures of

vetch and pea, the two legumes grown commonly between these sites. The highest rate of %Ndfa detected overall, however, was in winter pea in the intercrop at Site 3 at 92.7%, and multiple factors likely contributed to this. Soils with higher pH favor the NH_4 form of N over the NO_3 form (Walworth, 2013) and, consistent with this, NH_4 comprised a greater fraction of total available N (NH_4-N + NO_3-N) at the more alkaline Site 3, while NO_3 was in greater proportion at the other sites. Site 3 also had relatively low levels of available soil N overall, plus active growth of a companion wheat crop that would have further reduced levels of available soil N throughout the growing season, elevating %Ndfa even more.

The high rate of legume %Ndfa at Site 3 helped to elevate the crop-wide rate of BNF at this site. Despite that just 59% of the total biomass at Site 3 was legume biomass, which was dominated by winter peas, the rate of BNF (54.6 kg N ha^{-1}) ranged from 74 to 82% of the rate of BNF (66.7 to 73.9 kg N ha^{-1}) in the monoculture peas at sites 1 and 2. It is interesting to point out that the rate of BNF varied little between the monoculture peas at Sites 1 and 2, with a numeric difference of just 7.2 kg N ha^{-1}, despite having larger differences in the factors that determine BNF (i.e. growth, %Ndfa). The situation was similar between the vetch at Sites 1 and 2, where BNF ranged from 45.8 to 51.3 kg N ha^{-1}, a numeric difference of just 5.5 kg N ha^{-1}. Given that Sites 1 and 2 differed in soil factors but not in weather factors (e.g. precipitation was equal due to proximity), the legumes seemed to utilize the soil resources available to them to maintain a similar rate of BNF by balancing site-specific differences in growth, percent N, and %Ndfa. Several reports on legumes suggest that dry biomass can be used to

predict BNF capacity (Zhang et al., 1995; Sato, 2014; Lira et al., 2015; Divito and Sadras, 2014; Unkovich et al., 2010), but the current results indicate such predictions could lack accuracy when comparing results across sites that differ in soil factors.

4.6. Conclusions

Among the tested legumes, in average weather conditions for the region, Austrian winter pea was among the best producers and seemed to have an advantage as a N-fixer, followed closely by hairy vetch. Lentil, which has received little research attention in the region, also proved to be a good producer and N-fixer, comparable to hairy vetch. Crimson clover did not perform well in the systems investigated here. When peas and vetch were compared at nearby sites, differing in soil but not weather factors, the legumes seemed to maintain a similar rate of BNF by balancing site-specific differences in growth, percent N, and %Ndfa that depended on soil resource availability. Wheat and peas can be productively intercropped as a hay crop in the region, with wheat contributing to increased legume %Ndfa by sequestering plant available soil N and encouraging BNF. Austrian winter peas, hairy vetch, and lentil, grown as cash or cover crops, can play a major role in sustainable agriculture in the Southern Great Plains region. Any of these legumes can be incorporated into regional cropping systems to fix N, reducing the need for chemical fertilizer applications, while providing other ecosystem services.

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5. CONCLUSIONS

Legumes grown as cash and cover crops are a valuable component of cropping systems due to their ability to fix atmospheric nitrogen through the process of biological nitrogen fixation (BNF). In the Southern Great Plains region of the United States and particularly in Texas, there is very little information regarding the N-fixing potential of legumes. In this thesis, three studies were carried out in order to contribute information on BNF and associated plant and management factors for legumes grown in this region.

The first study illustrated the benefits of inoculating guar in order to increase the capacity of the crop for BNF. In particular, USDA Rhizobium strains 3386 and 3089 positively influenced guar nodule parameters, nitrogen assimilation, and ultimately biomass and protein production. Only one commercial inoculant is currently available for the crop; therefore, this work can assist in increasing rationale and support for more research in this area.

The second study illustrated the temporal and spatial dynamics in root system physical and architectural traits of guar, including dynamics in nodule growth and senescence over 80 days. Before this research, very little information was known regarding belowground characteristics and functions of this legume. These results show the root system architecture for guar includes a deep proliferative root system with a single tap root and many fine lateral roots. Furthermore, nodules were concentrated toward the soil surface and the average nodule weight also decreased with soil depth.

This information is critical for understanding the drought tolerance and BNF capacity for guar.

The third study aimed to quantify biological nitrogen fixation of winter legumes in the Southern Great Plains. Out of all the species assessed in this study, pea appeared to be the strongest nitrogen fixing legume based on its consistently high %Ndfa and total BNF values. Vetch also did well with similar biomass and nitrogen content. Lentil was comparable to vetch with moderate biomass and high %Ndfa. Clover could not compare even though it had high %Ndfa, its low biomass resulted in a low rate of BNF. Overall, this research provides novel BNF data for winter legumes in this region.

The information this thesis provides is critical in understanding the role legumes can play in sustainable agriculture in the region and can help promote the production of legumes to producers as cash and cover crops in Texas and the Southern Great Plains.
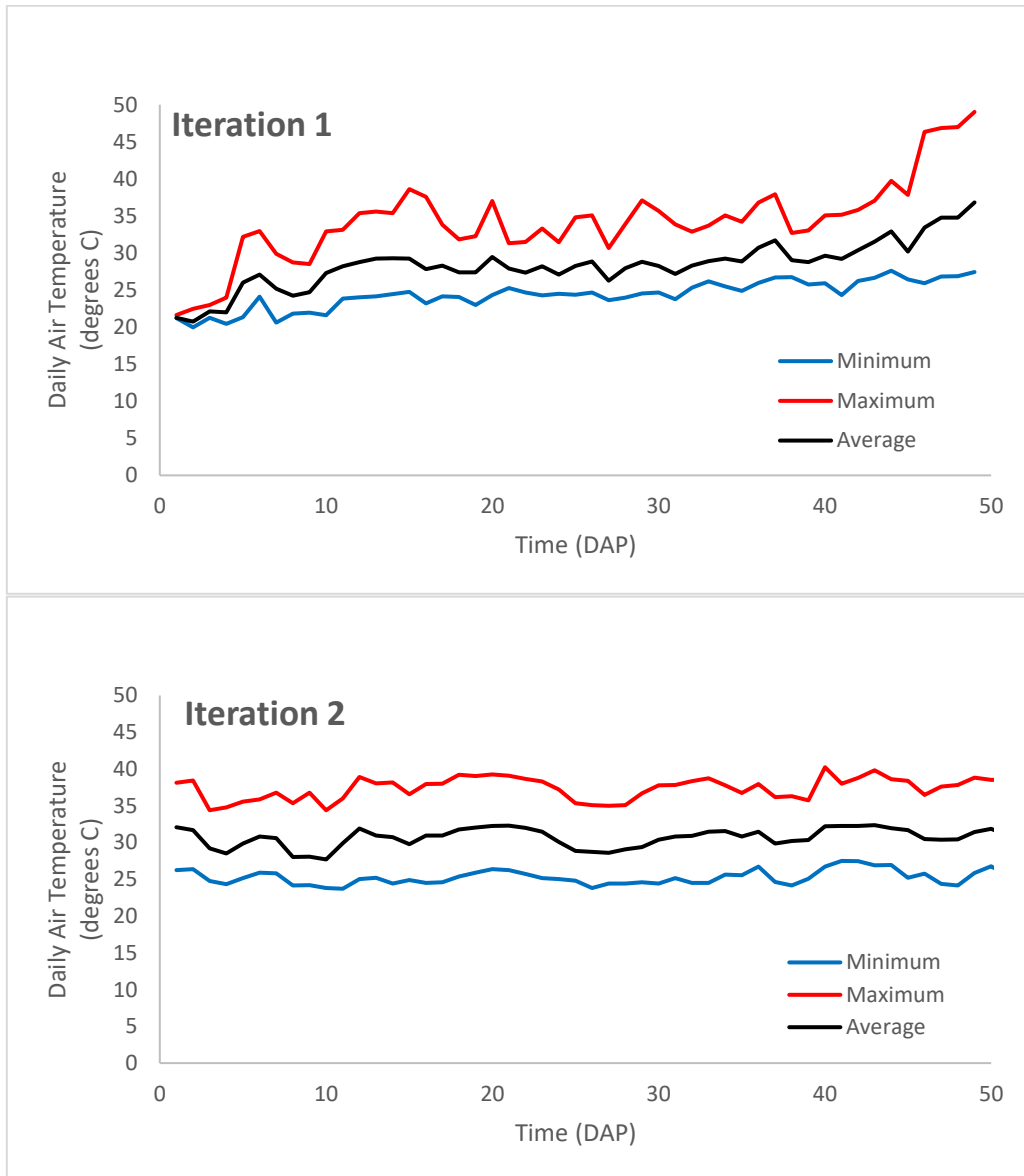
Figure 1.1. Maximum, minimum, and average daily ambient air temperatures in the greenhouse during both iterations of the study.
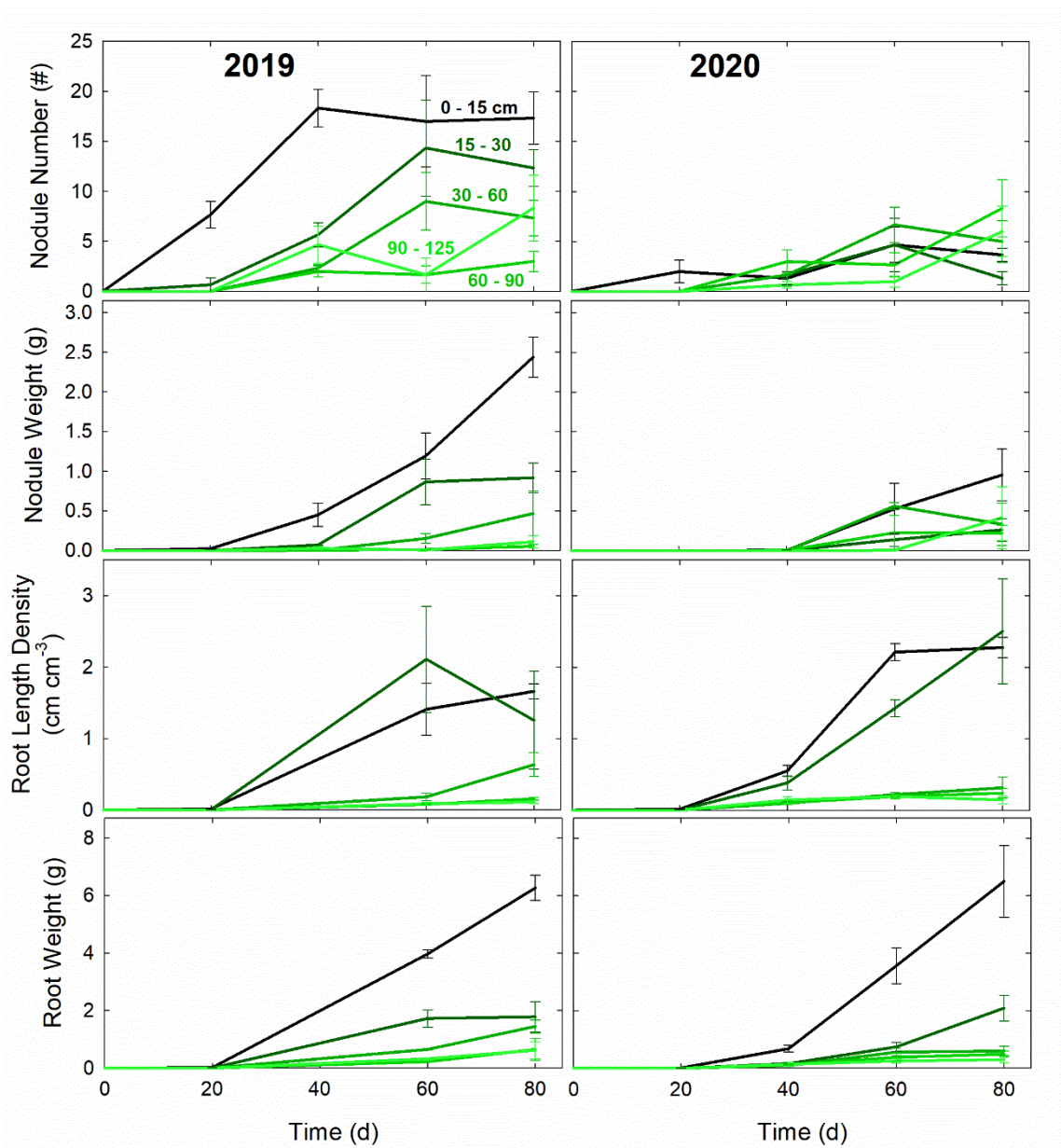
Figure 2.1. Changes in belowground root and nodule variables over time (80 days) and space (5 depth ranges). The error bars represent the standard error of the mean.
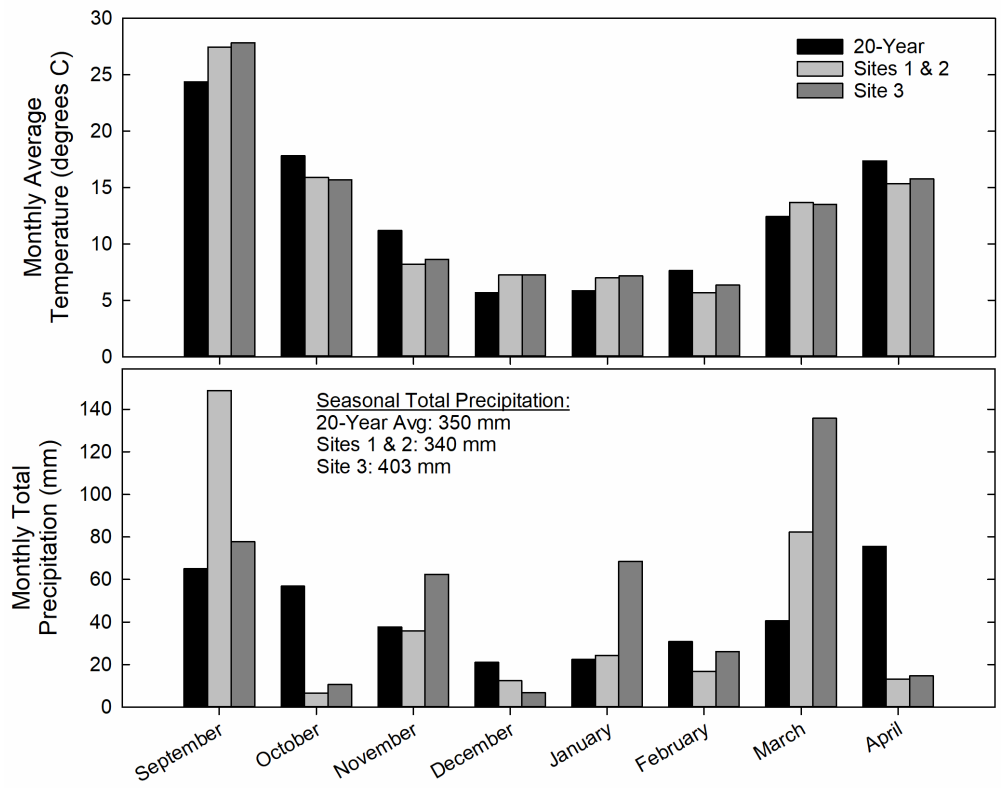
Figure 3.1. Weather data for Sites 1, 2, 3, as well as a 20-year average for the region.

TABLES

Table 1.1. Company names, scientific references, and inoculant information for global Rhizobium/Bradyrhizobium strains tested as inoculants for guar.

Company Name/Scientific Reference	Inoculant IDs	Company/Experiment Location	Commercially Available (Y/N)
USDA - ARS	USDA 3089, 3385, 3386, 3595	National Rhizobium Germplasm Collection	N
BASF, Australia *Gresta et al., 2019*	CB3035	Victoria, Australia *Modica, Italy*	Y
Chaudhary and Sindhu, 2016	HCS5, 36, 43, GSA11	*Hisar, India*	Unknown
Elnesairy et al., 2016	USDA 3089, 3385, 3386, ENRRI 16A	*Wad Medani, Sudan*	Unknown
NifTAL Center *Ibrahim et al., 2016*	TAL169, Hi12, and 12 locally isolated strains	Paia, Hawaii, USA *North Kordofan State, Sudan*	Unknown
Khandelwal and Sindhu, 2012	GSA3, 6, 11, 61, 74, 106, 110, 114, 115	*Hisar, India*	Unknown
NifTAL Center *Elsheikh and Ibrahim., 1998; Ibrahim et al., 2010 and 2011*	TAL169, 1371, ENRRI 16A, 16C	Paia, Hawaii, USA *Khartoum, Sudan*	Unknown
Yadav et al., 1984	5A/70, Tal174, 169, GR4	*Hisar, India*	Unknown

Table 1.2. Measurement of physical and chemical properties for the soil in study 1.

Parameter	mg/kg
NO_3-N	10.03
NH^4-N	2.05
P	28
K	228
Mg	212.5
Ca	1166
S	22
B	0.45
Zn	2.2
Mn	64
Fe	60.5
Cu	2.95
pH	7.2
Organic Matter	0.68%

Table 1.3. The means of four aboveground plant parameters in response to six inoculant treatments and an uninoculated control. The *P*-values are the result of statistical contrasts, comparing the inoculants to the control individually and collectively.

Inoculant	Plant Height		Total Nodes		Fruiting/Flowering Nodes		Biomass	
	(cm)	(*P*-Value)	(#)	(*P*-Value)	(#)	(*P*-Value)	(g)	(*P*-Value)
Control	47.2	-	17.0	-	16.5	-	18.8	-
USDA 3089	48.7	0.6395	18.5	0.2363	18.8	0.4091	22.2	**0.0155**
USDA 3385	46.1	0.5178	18.1	0.4674	15.9	0.8179	21.1	0.0968
USDA 3386	46.1	0.7169	18.4	0.3018	13.6	0.2928	23.2	**0.0022**
USDA 3595	47.9	0.8194	18.8	0.1377	15.0	0.5812	20.7	0.1656
Mix	47.1	0.9577	17.4	0.8400	13.8	0.3140	19.5	0.6187
All	47.1	0.9245	18.0	0.2848	15.6	0.6014	20.9	**0.0197**

Table 1.4. Mean nodule number and weight per plant in response to six inoculant treatments and an uninoculated control. The *P*-values are the result of statistical contrasts, comparing the inoculants to the control individually and collectively.

Inoculant	Total Nodules		Nodule Weight	
	(#)	(*P*-Value)	(g)	(*P*-Value)
Control	17.1	-	0.861	-
USDA 3089	18.6	0.7553	1.01	**0.0362**
USDA 3385	26.1	0.0669	1.05	**0.0121**
USDA 3386	21.6	0.3521	1.11	**0.0009**
USDA 3595	18.1	0.8354	0.95	0.2286
Mix	17.1	1	1.01	**0.0433**
All	19.8	0.3926	1.00	**0.0062**

Table 1.5. Means of four plant chemical composition parameters in response to six inoculant treatments and an uninoculated control. The *P*-values are the result of statistical contrasts, comparing the inoculants to the control individually and collectively.

Inoculant	Percent Carbon		Percent Nitrogen		Carbon to Nitrogen Ratio		Total Assimilated Nitrogen	
	(%)	(*P*-Value)	(%)	(*P*-Value)	(g g^{-1})	(*P*-Value)	(g)	(*P*-Value)
Control	38.6	-	2.72	-	14.7	-	0.53	-
USDA 3089	38.7	0.6121	2.88	0.2401	13.6	0.1878	0.65	**0.0077**
USDA 3385	38.8	0.4300	2.75	0.7762	14.3	0.5950	0.58	0.1889
USDA 3386	38.9	0.2236	2.76	0.7241	14.3	0.6041	0.65	**0.0076**
USDA 3595	38.6	0.7957	2.73	0.9025	14.2	0.5780	0.57	0.3313
Mix	38.5	0.4546	2.57	0.3003	15.4	0.4464	0.50	0.5278
All	38.7	0.6952	2.74	0.8158	14.4	0.5749	0.58	0.0668

Table 1.6. Percent protein and protein production means in response to six inoculant treatments and an uninoculated control. The *P*-values are the result of statistical contrasts, comparing the inoculants to the control individually and collectively.

Inoculant	Percent Protein		Protein Production	
	(%)	(*P*-Value)	(g)	(*P*-Value)
Control	15.9	-	3.10	-
USDA 3089	16.9	0.2397	3.79	**0.0077**
USDA 3385	16.2	0.7754	3.43	0.1886
USDA 3386	16.2	0.7227	3.79	**0.0076**
USDA 3595	16.0	0.9028	3.34	0.3314
Mix	15.1	0.3008	2.94	0.5282
All	16.1	0.8149	3.46	0.0667

Table 2.1. Measurement of physical and chemical properties for the soil in study 2.

Parameter	2019 (mg kg^{-1})	2020 (mg kg^{-1})
NO$_3$-N	10	5.5
NH^4-N	2.1	0.38
P	28	33
K	228	204
Mg	213	144
Ca	1166	915
S	22	9
B	0.45	0.50
Zn	2.2	3.2
Mn	64	54
Fe	61	52
Cu	3.0	1.2
pH	7.2	7.5
Organic Matter	0.68%	0.21%

Table 2.2. The means for four aboveground plant parameters every 10 days after planting in 2019 and 2020. The error term is the standard error.

Time (d)	Height (cm)	Nodes (#)	F/F Nodes (#)	Biomass (g)
2019				
10	5.8 ± 0.23	1.0 ± 0.0	0	**-**
20	8.1 ± 0.34	2.5 ± 0.15	0	0.34 ± 0.086
30	16 ± 1.0	6.7 ± 0.29	0	**-**
40	30 ± 1.3	11 ± 0.34	4.8 ± 0.43	19 ± 1.6
50	51 ± 3.7	20 ± 0.96	26 ± 3.3	**-**
60	65 ± 5.2	25 ± 1.1	50 ± 6.0	73 ± 9.1
70	88 ± 13	33 ± 2.0	50 ± 14	-
80	97 ± 16	37 ± 3.4	130 ± 4.0	167 ± 36
2020				
10	3.9 ± 0.14	1.0 ± 0	0	-
20	6.2 ± 0.24	1.2 ± 0.11	0	0.16 ± 0.012
30	16 ± 1.2	6.9 ± 0.39	1.7 ± 0.37	-
40	32 ± 2.2	12 ± 0.41	6.0 ± 0.82	7.0 ± 1.4
50	62 ± 4.1	20 ± 1.3	21 ± 1.8	-
60	82 ± 6.9	24 ± 2.1	31 ± 2.0	60 ± 5.1
70	93 ± 14	25 ± 1.5	47 ± 4.9	-
80	94 ± 12	25 ± 1.5	54 ± 6.1	135 ± 37

Table 2.3. The means of nodule parameters, integrating the entire depth of the columns, for each harvest interval in 2019 and 2020. The error term is the standard error.

Time (d)	Nodules (#)	Nodule Mass (g)
2019		
20	8.3 ± 1.8	0.025 ± 0.0077
40	33 ± 2.3	0.56 ± 0.13
60	44 ± 9.7	2.2 ± 0.089
80	48 ± 6.2	4.0 ± 0.42
2020		
20	2.0 ± 1.2	0.0010 ± 0.00058
40	8.0 ± 1.7	0.021 ± 0.011
60	20 ± 3.0	1.5 ± 0.44
80	24 ± 4.1	2.2 ± 0.28

Table 2.4. The means of root parameters, integrating the entire depth of the columns, for each harvest interval in 2019 and 2020. The error term is the standard error.

Time (d)	RLD (cm/cm^3)	Root Diameter (mm)	Root Mass (g)	Root/Shoot (g g^{-1})
2019				
20	0.0020 ± 0.00027	0.31 ± 0.012	0.021 ± 0.044	0.063 ± 0.0026
40	0.17 ± 0.016	0.46 ± 0.0041	2.3 ± 0.449	0.12 ± 0.015
60	0.51 ± 0.13	0.36 ± 0.011	6.9 ± 0.390	0.099 ± 0.019
80	0.57 ± 0.12	0.38 ± 0.024	11 ± 1.47	0.067 ± 0.0086
2020				
20	0.0014 ± 0.000049	0.30 ± 0.020	0.0087 ± 0.00067	0.055 ± 0.0071
40	0.19 ± 0.028	0.38 ± 0.0033	1.2 ± 0.219	0.17 ± 0.028
60	0.64 ± 0.029	0.37 ± 0.023	5.5 ± 1.108	0.090 ± 0.010
80	0.79 ± 0.17	0.44 ± 0.029	10 ± 1.395	0.080 ± 0.015

Table 2.5. Percent of root per diameter size class (mm) and specific root length (SRL), per soil depth, for the last harvest interval (80 DAP) in 2019 and 2020. The error term is the standard error.

Depth (cm)	0-0.5 mm (%)	0.5-1.0 mm (%)	1.0-2.0 mm (%)	2.0-3.0 mm (%)	> 3.0 mm (%)	SRL (m mg^{-1})
2019						
0-15	77.3	15.8	5.48	0.856	0.564	13 ± 1.1
15-30	87.1	10.7	1.77	0.090	0.340	33 ± 11
30-60	85.3	12.2	1.99	0.237	0.273	48 ± 16
60-90	90.4	6.97	1.52	0.200	0.910	45 ± 19
90-125	85.1	10.2	3.13	0.764	0.806	32 ± 13
2020						
0-15	72.7	19.4	6.41	0.916	0.574	19 ± 4.2
15-30	64.2	21.7	10.3	2.21	1.59	56 ± 5.0
30-60	75.8	16.9	5.31	1.10	0.890	60 ± 26
60-90	78.5	16.4	3.80	0.783	0.517	49 ± 11
90-125	86.9	9.07	2.84	0.372	0.818	54 ± 25

Table 2.6. Regression analysis of trends in eight above- and belowground plant parameters over time in 2019 and 2020.

Variable	Coefficient	Intercept	Relationship	R^2	*P*-value
2019					
Plant Height	0.0132	-0.642	Quadratic	0.91	<0.0001
Node Number	0.00370	-2.60	Quadratic	0.96	<0.0001
F/F Node Number	0.0486	50.6	Quadratic	0.86	<0.0001
Biomass	2.77	-73.8	Linear	0.78	0.0001
Nodule Number	0.653	0.667	Linear	0.69	0.0008
Nodule Mass	0.000762	-0.167	Quadratic	0.95	<0.0001
RLD	0.0102	-0.201	Linear	0.73	0.0004
Root Mass	0.998	-2210	Quadratic	0.93	<0.0001
2020					
Plant Height	0.00774	-11.1	Quadratic	0.88	<0.0001
Node Number	-0.000883	-6.15	Quadratic	0.90	<0.0001
F/F Node Number	0.00606	-19.5	Quadratic	0.93	<0.0001
Biomass	2.29	-63.8	Linear	0.72	0.0005
Nodule Number	0.393	-6.17	Linear	0.82	<0.0001
Nodule Mass	0.0402	-1.09	Linear	0.78	0.0001
RLD	0.0141	-0.302	Linear	0.83	<0.0001
Root Mass	2.060	-264.5	Quadratic	0.90	<0.0001

Table 3.1. Means and statistical analysis of plant productivity and N-fixation parameters for cover crops at Site 1. (Ndfa, percent of N derived from the atmosphere; Total BNF, total fixed N per unit area).

Species	Biomass (Mg ha^{-1})	N Content (%)	Ndfa (%)	Total N (kg N ha^{-1})	Total BNF (kg BNF ha^{-1})
Winter Pea	2.73 ab	3.29 a	72.6 a	90.2 a	66.7 a
Hairy Vetch	2.82 a	3.30 a	54.7 b	92.8 a	51.3 a
Crimson Clover	.497 c	2.29 b	52.6 b	11.7 b	6.08 b
Rye	1.95 b	.980 c	-	19.3 b	-
P-Value	*0.0001*	*< 0.0001*	*0.0532*	*< 0.0001*	*0.0012*

Table 3.2. Means and statistical analysis of plant productivity and N-fixation parameters for cover crops at Site 2. (Ndfa, percent of N derived from the atmosphere; Total BNF, total fixed N per unit area).

Species	Biomass (Mg ha^{-1})	N Content (%)	Ndfa (%)	Total N (kg N ha^{-1})	Total BNF (kg BNF ha^{-1})
Winter Pea	3.44 b	3.72 a	57.2 a	128 a	73.9 a
Hairy Vetch	3.56 b	3.02 b	34.4 b	109 ab	45.8 b
Lentil	2.54 c	3.63 a	51.8 a	91.8 bc	48.6 b
Rye	5.09 a	1.44 c	-	70.9 c	-
P-Value	*<0.0001*	*<0.0001*	*0.0048*	*0.0056*	*0.0321*

Table 3.3. Means ± standard deviation of plant productivity and N-fixation parameters for cash crops at Site 3. (Ndfa, percent of N derived from the atmosphere; Total BNF, total fixed N per unit area).

Species	Biomass (Mg ha^{-1})	N Content (%)	Ndfa (%)	Total N (kg ha^{-1})	Total BNF (kg ha^{-1})
Legumes	1.79 ± 0.71	3.21 ± 0.33	92.7 ± 3.74	58.6 ± 17.5	54.6 ± 15.5
Wheat	1.22 ± 1.8	1.18 ± 0.070	-	14.7 ± 10.9	-
Total	3.01 ± 0.34	2.46 ± 0.58	56.3 ± 16.9	72.6 ± 8.93	54.6 ± 15.5

Table 3.4. Measurement of physical and chemical properties for the soil in study 3.

Parameter	Site 1	Site 2 (mg kg $^{-1}$)	Site 3
Total N	1095	2420	1471
NO_3-N	8.53	17.34	0.790
NH_4-N	0.940	2.37	5.78
P	38.0	46.0	15.5
K	239	532	214
Mg	231.5	359	744.5
Ca	1510	2982	5100
S	6.5	7.5	25.5
B	1.3	0.9	1.35
Zn	1.0	1.95	0.70
Mn	90.5	137	148
Fe	54.0	49.5	26.5
Cu	1.4	2.15	1.5
pH	6.6	6.9	8.2