EXPLORING THE GENETIC RESOURCES OF *LENS* AND *RHIZOBIUM* TO IMPROVE THE BIOLOGICAL NITROGEN FIXATION (BNF) ABILITY IN THE LENTIL CROP

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By

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ABSTRACT

Lentil plants (*Lens culinaris*) have the ability to obtain most of the N they need from N fixation by establishing an efficient symbiotic relationship with *Rhizobium*. Plant-based diets are gaining the recognition they deserve for sustainability and producing legumes without the use of synthetic N fertilizers is the most sustainable approach. The N fixing ability of representative lentil cultivars, as well as accessions from 6 wild *Lens* species, was evaluated to determine the potential for wild germplasm to contribute positively to breeding for improved BNF. The contributions of diverse *Rhizobium* strains from 5 species to lentil productivity under local field conditions was also investigated. Subsequently, the level of specificity of the interactions between *Lens* accessions and *Rhizobium* strains with desirable N fixing abilities was explored. How traits related to N fixation are inherited was determined in three interspecific RIL populations from parents displaying contrasting phenotypes. Differential N fixing ability was found among cultivars and wild accessions; no particular species stood out. Wild accessions exhibited indeterminate nodulation, root modifications that responded to different N sources, higher seed percentage protein content, and yields comparable to plants fertilized with synthetic N. CDC Greenstar was the only cultivar with similar yield when inoculated or fertilized. CDC Maxim inoculated with the strain NZLR-24 (*Rhizobium leguminosarum* bv. *viciae*) had 9% higher yield under field conditions compared to when inoculated the commercial strain BASF 1435 (*Rlv*) and 15% more compared to a non-inoculated treatment. Some wild accessions demonstrated a promiscuous ability to efficiently fix N with a broad set of strains, but no cultivar did. The higher effective capacity of the strain NZLR-24 (*Rlv*) was also evident when used to inoculate *Lens* from 6 species, making it a suitable option for improved inoculants in the Northern Great Plains, as well as showing its value for selection in future breeding efforts. The strain Oyali B (*Rlv*) was also noteworthy for its superior interaction with wild *Lens,* and it is an attractive wild-type resource. Sixteen QTL were identified for nodulation traits among the three interspecific populations; eight were meta-QTL found across two or more populations. Chromosomes 1 and 6 had Meta-QTL for number of nodules, nodule weight and specific nodule weight. Chromosome 7 had one for specific nodule weight. This study establishes the necessary groundwork for understanding the role that exotic germplasm can play for the breeding of better N fixation ability in the lentil crop.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BNF	Biological nitrogen fixation
C	Control
cM	CentiMorgan
CV	Coefficient of variation
DTF	Days to flower
DTM	Days to maturity
DAS	Days after sowing
H^2	Broad sense heritability
HI	Harvest index
ICIM	Inclusive composite interval mapping
KSW	Thousand seed weight
L	liter
LG	Linkage group
LS-mean	Least squares mean
LSD	Least significant difference
LOD	Log-of-odds
Mha	Million hectares
Mt	Million tonnes
N	Nitrogen (elemental)
N_2	Dinitrogen
+N	Added N fertilizer
NC	Nodule colour
Ndfa	Nitrogen derived from the atmosphere
NDW	Nodule dry weight
NFW	Nodule fresh weight
NH_3	Ammonia
NN	Number of nodules
NN1	Number of nodules from 0-15 cm depth
NN2	Number of nodules from 15-25 cm depth
PVC	polyvinyl chloride
PVE	phenotypic variation explained
PY	Protein yield
QTL	Quantitative trait loci
R	*Rhizobium*
RAD	Root average diameter
RCBD	Randomized complete block design
RDW	Root dry weight
RFW	Root fresh weight

RIL	Recombinant inbred line
Rlv	*Rhizobium leguminosarum* bv. *viciae*
RNY	Root N yield
R:S ratio	Root to shoot ratio
SD	Standard deviation
SDW	Shoot dry weight
SFW	Shoot fresh weight
SE	Standard error
SN	Seed number
SNP	Single nucleotide polymorphism
SNY	Shoot N yield
SpNFW	Specific nodule fresh weight
SpNDW	Specific nodule dry weight
SW	Seed weight
TNDW	Total nodule dry weight
TNN	Total number of nodules
TRL	Total root length
TRV	Total root volume
TRSA	Total root superficial area
YMB	Yeast mannitol broth
YMA	Yeast mannitol agar

1. GENERAL INTRODUCTION

1.1. Background of the study

Lentil (*Lens culinaris* Medik.) is among the earliest crops that humans domesticated (Ladizinsky 1993). Lentil is a great resource for today's agriculture in the context of poverty, a growing population, the need for sustainable production systems and climate change (Suryapani *et al*., 2012). It is also a great source for human nutrition for its protein and micronutrients content including vitamins and minerals (Wang and Daun 2006; Thavarajah *et al*., 2009; Thavarajah *et al*., 2011; Kumar *et al*., 2016). It represents an attractive source for people choosing plant-based diets and people with restricted diets for its low-glycemic index (Campos-Vega *et al*., 2010).

It is the sixth most produced legume (Erskine *et al*., 2011) cultivated around the world in regions currently classified in three macro-environments: temperate region, Mediterranean and sub-tropical savannah (Khazaei *et al*., 2016). India is the largest consumer and Canada is the largest producer (FAO 2019).

Lentils are classified into three market classes: red, green and specialty types. There have been more than 40 varieties developed by the University of Saskatchewan, Canada, from the different market classes that provide high yielding and marketable seed characteristics. It has been recognized that the preservation and reintroduction of wild related germplasm is crucial for meeting the global food production demands for the present and the future (Global Crop Trust 2020). Interspecific hybridization can generate phenotypic novelty, of traits that are no longer present in the cultivated pool, which has been attempted in more than 29 crops (Maxted and Kell 2009).

Nitrogen (N) is often limiting in agricultural systems, but lentils have the ability to establish symbiotic relations with rhizobia bacteria to fix N. The biological nitrogen fixation (BNF) process is the second most important biological process in plants after photosynthesis, and the most important natural process for obtaining N in all legumes. Through this process, the bacteria convert atmospheric N_2 to N available for the plant, NH_3, in a symbiotic process that provides carbohydrates and a favorable environment in exchange. BNF not only contributes to agricultural systems by providing N to crops in forms that are then directly harvested for human consumption as fresh vegetables and dried grain (Salvagiotti, *et al*., 2008), but also leaves below ground contributions of N for subsequent crops (Aslam *et al*., 2003).

Rhizobium is one of the six genera of bacteria that are known to nodulate legumes. The symbionts of lentils are strains from the species *Rhizobium leguminosarum* bv. *viciae*. Several other *Rhizobium* species are known to nodulate lentil as a product of local adaptation events (Harun-or Rashid *et al*., 2014; Harun-or Rashid *et al*., 2012; Taha *et al*., 2018).

About 20 Mt of N fertilizers are used in North America every year (IFA 2014). Price has tripled since 2005 and demand increases about 5% annually (IFA 2014) directly affecting producers' profits. BNF is a suitable alternative to synthetic fertilizer in the lentil crop, with average values of % nitrogen derived from the atmosphere (%Ndfa) of 63% that range from 28-87% (Herridge *et al*., 2008) according to a number of factors directly and indirectly related to the symbiotic relationship. Legume-based rotational systems were adopted in the Northern Great Plains about 50 years ago (Spratt *et al*., 1975) to improve soil conditions that had deteriorated after 80 years of fallow-based wheat cropping (Schnitzer *et al*., 2006).

The potential contributions from legumes to cropping systems range from 45-75% of the N fixed (Walley *et al*., 2007) and further benefits such as hydrogen emitted in the N fixing process that impact the soil microbiota have been also measured (Salvagiotti *et al*., 2008). It is also known that most estimates have ignored below ground values, which have proven to represent a significant contribution (Arcand *et al*., 2014). Yield enhancement has been observed in wheat in legume-based systems (Kirkegaard *et al*., 2008; Aslam *et al*., 2003). In the Northern Great Plains, an established rhizobia population is present after years of inoculation (Lupwayi and Kennedy 2007) and the persistence of this population depends on the rotational programs used by farmers (Chemining'wa and Vessey 2006). However, inoculation is always recommended to maximize yield results (Vessey 2004), and benefits have been reported in the Northern Great Plains on commercial farms (Lupwayi and Kennedy 2007).

The success of a symbiotic relationship involves the complex role of several direct and indirect agro-environmental factors. Direct factors include the plant genotype and the bacterial strain. Indirect factors include but are not limited to interactions with the soil microbiota, soil nutritional composition, soil structure, biotic and abiotic factors. The complexity of indirect factors influencing the success of this relationship requires local experimentation under the target agro-environmental conditions (Ruiz-Diez *et al*., 2012a). Increasing the efficiency of N fixation requires selecting for higher efficiency among genotypes and strains, but also for the best

combination for nodulation effectiveness, given the level of specificity in this relationship (Wang *et al*., 2012). Both higher performing strains of *Rhizobium leguminosarum* bv. *viciae* and the most predominant lentil cultivars available at the time have been tested under local conditions in the Northern Great Plains (Abi-Ghanem *et al*., 2011, Bremer *et al*., 1990). As new lentil varieties emerge, wilds are introgressed into the breeding program, and new *Rhizobium* strains are characterized, this has to be revised.

Breeding has successfully led to increased yield and improved seed characteristics; increasing the production of lentils but also narrowing the genetic variability, that has consequently reduced the levels of resistance to biotic and abiotic factors (Erskine *et al*., 1998). There are six wild relative species in the genus *Lens* (Wong *et al*., 2015) and several have been used for introducing useful genetic variation into the domesticated species genome. The most important progress has been in the identification of sources for disease resistance such as anthracnose, ascochyta blight and stemphylium blight (Vail *et al*., 2012; Podder *et al*., 2012; Gupta and Sharma 2006). For symbiotic related traits, it its known that modern varieties are usually selected under high fertility conditions, provided by chemical fertilizers, making it unnecessary for the plant to establish effective interactions with symbiotic soil microorganisms, contributing to the loss of genes that control these traits (Wissuwa *et al*., 2009). Higher symbiotic ability has been successfully introgressed into common bean (*P. vulgaris*) from its relative tepary bean (*P. acutifolius*) (Somasegaran and Hoben 1991). The opposite has been reported in soybean (*Glycine max*) for which higher symbiotic ability seem to be an ability gained during domestication (Munoz *et al*., 2016).

Some of the most important benefits of improving legume N fixation include increasing the amount of Ndfa, thereby reducing the requirements for chemical N fertilizers, increasing legume yields, and contributing to below ground N available to subsequent crops, all of which are major contributors to more sustainable agricultural systems and reducing the impact of agriculture to the environment.

1.2. Hypotheses

1. There are superior species/accessions that can contribute positive N fixation related alleles to modern lentil varieties.

2. Genetically diverse *Rhizobium* from the center of origin of lentils and other main production areas, will allow the incorporation of more efficient strains to the lentil cropping system in the Northern Great Plains.

3.There is symbiotic specificity at the strain × accession level among *Lens* and *Rhizobium* species.

4. Specific regions of the wild and cultivated genomes associated with N fixation related traits can be identified allowing breeders to develop strategies to access variability from wild lentil species.

1.3. Objectives

1.Identify wild species/genotypes within the genus *Lens* that can contribute alleles responsible for a higher symbiotic ability to cultivated lentil (*L. culinaris*).

2. Identify specific strains of *Rhizobium* that can adapt well to the local agroecosystem conditions to improve BNF in lentil in the Northern Great Plains.

3. Establish the effectiveness of 10 genotypes from 6 *Lens* species in combination with 4 *Rhizobium* strains from 3 species to better understand specificity of their relation at the strain × accession level.

4. Conduct a phenotypic characterization of the interspecific populations LR-68, LR-70 and LR-86 for symbiotic related traits when inoculated with the strain NZLR-24 (*Rlv*) and identify QTL associated with nodulation characteristics in these populations.

1.4. Expected contributions

A better understanding of the differential ability in N fixation among *Lens* species, and the contributions of diverse *Rhizobium* species to symbiotic efficiency, will be obtained from this study, as well as the level of specificity of this relationship. It will also provide knowledge on how symbiotic related traits are inherited in interspecific populations and the genomic regions for some nodulation traits. It will represent a pre-breeding tool for the future breeding for better BNF ability in the lentil crop, setting the groundwork for a better understanding of the role of exotic germplasm for improving BNF.

2. LITERATURE REVIEW

2.1. The lentil

2.1.1. General aspects of lentils

Lentil (*Lens culinaris* Medik.) is a cold-season grain legume and one of the oldest domesticated legumes (Harlan 1992) often cultivated under limiting agricultural conditions and environments. It was among the earliest plants domesticated in Southwest Asia (Ladizinsky 1993) and spread to different regions of the world from there. Currently, production regions are classified in three macro-environments: temperate region (e.g., northern great plains of Canada and the USA), Mediterranean (e.g., Turkey, Spain, Morocco) and sub-tropical savannah (e.g. Nepal, India and Bangladesh) (Khazaei *et al*., 2016) which are characterized by different temperature and photoperiod regimes during the growing season.

Lentil is the sixth in the world in terms of production among the pulse crops (FAOSTAT 2019). Lentil production plays a key nutritional role in developing countries and also in the economy of developed ones. Lentils have been part of the diet and crucial source of protein in many of the west Asian countries where lentil originated, including Turkey and Syria as well as India, Bangladesh and Nepal in South Asia and other countries in Africa and the Americas including Eritrea, Algeria, and Colombia. India is the largest consumer worldwide (FAO 2019). Lentil was first introduced to Canada in 1970 as a rotation crop, and it was best adapted to Saskatchewan (Carew *et al*., 2013). Canada, primarily Saskatchewan, became the largest producer and exporter over the last two decades, with a total production of 19 Mt from 1998 to 2014. In 2015, Canadian lentil production reached 3.2 Mt (FAO, 2016).

Progressively, it is also becoming part of the diet in developed countries due to its known health benefits (James and Major 2002). It is an important source of protein for humans - containing up to 30% seed protein content (Wang and Daun 2006). About 50% of the composition are carbohydrates, with a higher level of dietary fiber in comparison to cereals (Joshi *et al*., 2012). Lentil also represents a good source of micronutrients, including vitamins (folate and riboflavin) and minerals (iron, selenium, and zinc) (Thavarajah *et al*., 2009; Thavarajah *et al*., 2011; Kumar *et al*., 2016). In addition to this, a low-glycemic index makes lentils a good option for people that require limited diets (Campos-Vega *et al*., 2010), as well as for people choosing plant-based

diets. The ability of lentils to efficiently fix N and adapt to a wide range of environments, as well as its impact on enhancing cereal yield on intercropping systems, makes it a sustainable crop (Suryapani 2012).

A broad range of lentil cultivars are grown and are classified into three market classes according to their colour and size. The red market class has orange or red cotyledons of extra-small, small or large size. The green market class has green seed coats and yellow cotyledon colour and can be small, medium or large. Typically, green market class lentils have larger seeds compared to the red market class. The third class is the specialty types and includes the Spanish Brown with gray dotted seed coats and yellow cotyledons, and the French green type with a marbled coat and yellow cotyledons, among others.

The lentil breeding program at the University of Saskatchewan plays a key role in western Canadian agriculture, providing varieties to meet production demands. In 1980 the varieties Eston and Laird were available. Currently, more than 40 varieties from different market classes have become available (SPG 2021). A key step was the Clearfield® system, that gives tolerance to imidazolinone herbicides. CDC Maxim is currently the most grown variety in North America (SPG 2020).

2.1.2. Genetic resources for lentil breeding: back to wilds

Domestication and breeding efforts have contributed to great progress in major seed traits and yield that are fundamental for food security, but these improvements negatively impacted traits that confer crop resistance to biotic, and abiotic stresses and several other characteristics in the most important cultivated species (Spillane and Gepts 2001; Olsen and Wendel 2013; Meyer *et al*., 2012).

Exploring the potential contributions of wild and related species to improved resilience in crops is now one of the focuses of many breeding programs to meet the demands of this century (Tester and Langridge 2010; Maxted and Kell 2009; McCough *et al*., 2013). Wild relatives are recognized as the greatest source of genetic diversity for traits no longer present in our cultivars. They are the key to adapting our current crops to current and future conditions and food security (Global Crop Trust 2020).

For years there have been obstacles and reluctance about using wild pools (McCouch *et al.*, 2013) due to linkage drag of undesirable characteristics. However, gains realized from interspecific hybrids including characteristics no longer present in domesticated crops have been demonstrated in several species. It is proposed that building robust collections of wild relatives and generating exotic genetic libraries (Zamir *et al.*, 2001) assisted by the latest genome sequencing techniques in combination with phenotyping for desire traits, will give domesticated crops the resilience needed for the current and future conditions (Warschefsky *et al.*, 2014). This is helping to close the gap in the actual use of genetic diversity collected for about a century (Tanksley and McCouch 1997).

The importance of broadening the genetic basis is recognized in lentil and diverse resources have been collected and included in breeding programs (Erskine *et al.*, 2011; Furman *et al.*, 2006). The International Center for Agricultural Research in the Dry Areas (ICARDA) preserves the biggest *Lens* germplasm collection available in the world of 10, 800 accessions, from which 583 are wild genotypes from 24 countries (Erskine *et al.*, 2011, Cubero *et al.*, 2009). The Australian Temperate Field Crops Collection (ATFCC), The United States Department of Agriculture (USDA) Agricultural Research Service (ARS) and All-Russian Research Institute of Plant Industry (VIR) host the other major collections worldwide (Furman *et al.*, 2009).

Plant species from the same genus are classified in gene pools as defined by Harlan and de Wet (1971). Species in the primary gene pool can cross freely and produce fertile hybrids. Species in the secondary pool cross but have a lower degree of fertility. Those in the tertiary gene pool can produce successful interspecies offspring only after using chromosome doubling, embryo rescue or other tissue culture techniques.

There are seven known species in the genus *Lens*, categorized in four gene pools based on their crossing compatibility and genetic similarity (Wong *et al.*, 2015; Cubero *et al.*, 2009). The primary gene pool includes the domesticated species *L. culinaris*, its progenitor species *L. orientalis*, and *L. tomentosus*. *L. lamottei*, *L. odemensis* are in the secondary, *L. ervoides* in the tertiary and *L. nigricans* in the quaternary (Wong *et al.*, 2015). Successful introgression has been conducted out to the tertiary gene pool, but no successful crosses have been obtained with *L. nigricans*.

The main contributions of wild lentil species to improved biotic stresses include identification and introgression of novel sources of resistance to *Colletotrichum truncatum* from *L. ervoides* (Vail *et al*., 2012; Tullu *et al*., 2006; Gela *et al*., 2021), resistance to ascochyta blight (Tullu *et al*., 2010), stemphylium blight resistance (Podder *et al*., 2012; Kant *et al*., 2017), tolerance to broomrape (Fernandez-Aparicio *et al*., 2009) and resistant to seed bruchids (Laserna-Ruiz *et al*., 2012). Differential agro-morphological and phenological traits have also been identified (Yuan *et al*., 2017; Gupta and Sharma 2006; Gupta and Sharma 2007; Tullu *et al*., 2001, Singh *et al*., 2014; Erskine *et al*., 1989, Hoffman *et al*., 1988) for which wild species have been recognized as a key to enhanced yield and adaptability.

Differential characteristics in seed composition of oligosaccharides and sucrose concentration have also been a focus of study in the wild species (Tahir *et al*., 2012; Chen 2018). Furthermore, wild *Lens* species have shown unique phenotypic mechanisms of root modifications to tolerate drought stresses by more efficiently reaching deeper water (Gorim and Vandenberg 2017).

At the University of Saskatchewan, diverse genotypic and phenotypic data on chickpea (*Cicer arietinum*), common bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), faba bean (*Vicia faba*) and lentil is available through the KnowPulse web portal, including diverse data from crop wild relatives that give access to genetic maps and phenotypic characteristics that facilitate breeding (Sanderson *et al*., 2019).

2.1.2.1. Background of the interspecific populations LR-68, LR-70 and LR-86.

Developing interspecific populations is key for reintroducing desired alleles. Several recombinant inbreed lines (RIL) populations have been developed at the Crop Development Centre (CDC) of the University of Saskatchewan. These populations have been genotyped and phenotyped for novel identification of resistance sources to various diseases and better understanding of domestication traits. Genetic maps were developed under the Application of Genomic Innovation in the Lentil Economy (AGILE) project and are available for QTL mapping association (https://knowpulse.usask.ca/search/genetic-maps).

The LR-68 is a bi-parental RIL population that was produced using IG 72643 (*L. orientalis*) and CDC Greenstar (*L. culinaris*). It consists of a total of 121 RILs. The LR-70 is a bi-parental RIL population whose parents are Eston (*L. culinaris*) and IG 72623 (*L. odemensis*) and consist of

119 RILs. Eston is the most cross compatible *L. culinaris* (Tullu *et al*., 2013) from the accessions attempted to cross. These populations were developed by the lentil breeding program at the University of Saskatchewan for the introgression of disease resistance and study of agronomic and seed traits. The LR-86 population was produced at the University of León using the Spanish cultivar Lupa and BGE 016880 (*L. orientalis*) and consists of 93 RILs (Fratini *et al*., 2007). The genetic components determining the regulation of flowering in response to light quality, as well as growth habit and yield have been studied in this population LR-86 (Fratini *et al*., 2007; Yuan *et al*., 2021).

2.2. Biological nitrogen fixation (BNF)

2.2.1. BNF in agriculture

Dinitrogen (N_2) gas constitutes about 80% of the atmosphere but is not directly available to plants. Biological N fixation (BNF) it is the process in which specific bacteria can convert atmospheric N_2 into ammonia (NH_3), a form of N that can be used by plants. It constitutes the second most important biological process in plants after photosynthesis. There are six main genera of bacteria that fix N when partnered with a legume: *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Azorhizobium*, *Mesorhizobium* and *Allorhizobium*, collectively known as rhizobia.

About 200 Mt (million tonnes) of mineral N are required annually to drive agriculture in the world; from those, approximately 20 Mt are used in North America (IFA 2017). Around 100 Mt are supplied via the industrial Haber-Bosch fixation process. BNF contributes 35 Mt of N in association with legume crops (Herridge *et al*., 2008; Unkovich *et al*., 2008). The demand for N fertilizer grows about 5% yearly in North America (FAO 2014) and prices have tripled since 2005 (IFA 2009). It is estimated that up to 2003, US$7-10 billion were saved annually on N fertilizers due to N fixed by legumes (Hardarson *et al*., 2003). Improving the efficiency of N use via N fixation is crucial for driving agriculture towards a more sustainable food production system for the planet.

Increasing N fixation efficiency in legume crops is fundamental to optimize their role in alleviating dependency to chemical N fertilizer but also to maximize additional benefits including maximizing the yield and protein levels of products that are directly harvested (seeds, pods, vegetative parts), and enriching N in residues for subsequent cereal crops. This leads to

higher profits for growers and a reduction in their agricultural footprint by reducing gaseous losses that contribute to global warming and contaminate watercourses.

N fixing plants can obtain added N absorbed through roots, fixed N from their bacterial symbiont, and organic N sources may be accessed from the soil (Lipson and Näsholm 2001). When plants engage in symbiosis, root growth is also affected. N fixation makes biomass allocation to roots independent of soil nitrogen, when efficient, fewer roots on the inoculated plant and more shoot biomass is allocated that translates into more efficient N acquisition (Markham and Zekveld 2007).

Contributions of N fixation for all important legumes in different systems have been extensively reviewed and summarized, with values of the percentage of the nitrogen derived from the atmosphere (%Ndfa) ranging within each crop (Unkovich and Pate 2000; Peoples *et al*., 2009). %Ndfa is not only determined by the legume and the strain, but also influenced by the available soil N. When high N content is available from mineral sources, % Ndfa can be as low as 0. Conversely, if there is low mineral N available and effective rhizobia is present, 100 % of the N could be obtained through fixation. A whole range of N fixation values can be observed depending on these two components (Unkovich and Pate 2000; Peoples *et al*., 2009).

In legume cropping systems, variations in the amount of N fixed are also a consequence of agro-environmental factors and agronomic practices, and the plant species and genotypes play a role in determining the composition of the microbial community in the rhizosphere (Berg *et al*., 2002; Mazzola and Gu 2002, Miethling *et al*., 2000). Superior results have been obtained when previous selections under local growing conditions were made (Giller 2002, Boddey *et al.*, 1997).

Basically, anything that affects photosynthesis will affect the success of the symbiotic process, which will include biotic and abiotic factors. Any agro-environmental factor present where the symbiotic process in taking place, will affect in some degree the success of this relation. Some of these effects that have been directly measure include the role of arbuscular mycorrhizae (Zarei *et al*., 2006), P-use efficiency (Singh *et al*., 2005), pests (Weigand *et al*., 1992), temperature, salinity and moisture (Kessel and Hartley 2000) and soil properties (Ruiz-Diez *et al*., 2012).

2.2.2. BNF contributions to cereal crops and soil health.

BNF can be of benefit for subsequent crops and soil health. This will be affected by the symbiotic components: the plant genotype, the strains and their interaction, but also by the soil microbiota, agri-environmental biotic and abiotic factors and agronomical management (Quinn *et al*., 2009). It can contribute to subsequent cereal crops and organic N in the soil (Herridge and Bergense, 1988; Zapata 1990). Even though much of the fixed N is removed for consumption, values from 35 to 120 kg N/ha have been accounted for succeeding crops (Ghost *et al*., 2007). In legume crops, this contributions account from 45-75% of the N fixed to the system (Walley *et al*., 2007). It is estimated that from the 20-22 tonnes of N fixed globally every year, 17 are removed in grains (Herridge *et al*., 2008). Even if the N remaining for subsequent crops is small, there are other benefits unrelated to direct use of N such as hydrogen emission from N fixation that impact the soil biology (Salvagiotti *et al*., 2008).

In the other hand, below ground contributions have been mostly ignored. Every tonne of shoot dry matter produced by legume crops can produce in average 30-40 kg of N (whole plant) (Peoples *et al*., 2009). The contributions on wheat (*Triticum aestivum*) have been measured under greenhouse conditions on subsequent treatments with canola (*Brassica napus*) and pea, with pea contributing 13.4% from below ground residues, compared to canola with 7%, establishing the importance of accounting for belowground contributions and legume-based rotations (Arcand *et al*., 2014).

Intercropping has been a practice in parts of America and South Asia for millennia. About half of the lentil crop grown in Bangladesh is produced using this practice (Sarker *et al*., 2004). The benefits of intercropping have been observed in association with cereal crops. In a lentil-barley (*Hordeum vulgare*) system in a temperate climate, N fixation enhanced N uptake without reducing N uptake from the soil by the cereal (Schmidke *et al*., 2004). Benefits in N uptake have been also observed for wheat when intercropped with chickpea for both crops (Gunes *et al*., 2007)

Yield can also be improved in non-legume crops when legume-based systems are included in the cropping system in wheat and cotton, with profitability benefits also reported (Shah *et al*., 2003, Aslam *et al*., 2003; Kirkegaard *et al*., 2008; Rochester *et al*., 2001).

2.2.3. BNF in the Northern Great Plains

Cropping in the Northern Great Plains changed to a legume-based system to reduce some of the negative impact in the soil associated with the fallow-based system (Spratt *et al*., 1975). It is estimated that about 50% of the soil organic matter was lost from the soils in southern Saskatchewan as a consequence of cultivating wheat for 80 years (Schnitzer *et al*., 2006).

The introduction and expansion of legume cropping systems in the Northern Great Plains have impacted the population of microorganisms in the soil and mechanisms of N use, since there was no native population of *Rlv* in this area. The establishment of the rhizobia population has allowed the multiple contributions of N fixation (Lupwayi and Kennedy 2007). Experiments in multiple sites to characterize the abundance and effectiveness of *R. leguminosarum* in the eastern prairies of Canada have been conducted through sampling pea plants. The most desirable nodulation traits were found from fields where commercial inoculant was applied intensively, and very negligible response was found in those fields without inoculation, or lower organic matter and more acid pH value (Chemining'wa and Vessey 2006). Some of the greatest results for BNF have been observed when new legumes are planted in areas with no native population of rhizobia and a high performing inoculant is introduced, since there is no competition of multiple undesirable strains to infect the plant (Kessel and Hartley 2000).

About 171 million kg N were fixed by legumes (peas, lentils, common beans and chickpeas) in the Canadian Prairies and 40 million kg N fixed in the USA by the same crops in 2004. These amounts of N fixed represented about 8% of the total N used in agriculture that year (Lupwayi and Kennedy 2007). Values of the Ndfa were also estimated from 38 field experiments, and were in average 60%, that vary from 9-88%, with amounts of 0-192 of total N fixed in kg/ha (Walley *et al*., 2007). These estimates were double the amount to those observed in Canadian farmlands 20 years before, mainly because of the increase in legume planted area from 9 to 15 Mha (Yang *et al*., 2010). *Rhizobium leguminosarum* bv. *viciae* strains have been selected in Canada as lentil and pea inoculants (Rennie and Hynes 1993). Performance of 200 strains from Nitrogen Fixation by Tropical Agricultural Legumes (Niftal-USA), International Center for Agricultural Research in the Dry Areas (ICARDA), Department of Agriculture of Australia, University of Manitoba, Manitoba, Canada and elsewhere, have been tested under greenhouse and field conditions in Saskatchewan, with the lentil varieties Eston and Laird, finding superior results with interactions with the strains

from Syria (Bremer *et al*., 1990), suggesting the importance of exploring a broad range of strains under local field conditions with the new commercial varieties.

In this area the persistence in the soil of *R. leguminosarum* populations depend on the rotation programs used by farmers. It has been observed than they can be 10 to 100 times larger in fields with a history of peas compared to those with a history of wheat or common beans (Kucey and Hynes 1989). Inoculant carriers have also been tested in the Northern Great Plains to determine best inoculation methods. In experiments with chickpea, it was suggested that granular inoculant, which is placed about 3-8 cm from the sown seed, produced the best benefits in yield compared to liquid treatments (Kyei-Boahen *et al*., 2002).

Given the cost efficiency of the inoculation practice, producers are advised to always inoculate for maximum yield responses (Vessey 2004). It should be considered by farmers as an insurance against N deficient crops that will translate into poor yields and lower income (Unkovich *et al*., 2008).

2.2.4. Inoculation and *Rhizobium* inoculants.

High quality rhizobial inoculants are available nowadays and farmers are experiencing the advantages of inoculating crops not only in the crop cycle but also for the overall management of their cropping systems. The practice of inoculation ensures that sufficient bacteria are available for the crop to efficiently fix N. Both when growing legumes outside their centre of origin or in their native ranges, it is crucial the inoculation of strains with higher symbiotic efficiency. When growing in the native ranges, plants have a better ability to be colonized by symbionts, opposite in introduced areas, they cannot be colonized as efficiently and compensate by adapting more root biomass, adapt to more specific microbial and are more susceptible to be colonize by parasitic (Shelby *et al*., 2016). However, large populations of diverse rhizobia can infect with ineffective results (Unkovich *et al*., 2008).

Field trials are necessary to understand if the efficiency of the native or resident population of rhizobia can be surpassed with inoculation. Such trials need to include non-inoculated as well as N fertilized controls. When large nodules with visible red coloration are present in the non-inoculated control, a large soil population of effective compatible rhizobia is present. If no nodules or small with ineffective appearance are obtained, there is either no population of

rhizobia capable of infecting, population is infective but not effective, or soil is rich in mineral N (Howieson and Dilworth 2016).

The majority of commercial inoculants are of three types: peat-based powder, liquid and granular. Inoculant must be in direct contact with the seed and early root to ensure the relation process (Walley *et al*., 2007). Studies with *Sinorhizobium meliloti* show that mobility of rhizobia *per se* is unlikely to happen (Caetano-Anollés *et al*., 1992). The microorganism require water to move in the soil. To increase the chances of rhizobia to colonize the plant, minimal counts of alive cells are required to be present in commercial inoculum, usually from a million to a billion cells per inoculated seed. Canada is within the countries that regulate quality of inoculants: minimal counts of cells and contaminants (Stephens and Rask 2000). Technically, a single *Rhizobium* cell can produce more than a billion descendants inside a nodule and one effective cell infecting the root legume can be sufficient (West *et al*., 2002).

Inoculant carriers support the live rhizobia cells and determine in great manner the quality of the inoculant. Some of the most common and commercialized include organic peat substrates (Hungria *et al*., 2005) that are applied to the seed coat and can deliver high counts of cells but must be applied on wet soils. Also, peat-based granules sown alongside with the seed, that has proven to be very efficient (Kyei-Boahen *et al*, 2002). There are also liquid inoculants, that are sprayed onto seed and have proven to be less robust than other methods. All are susceptible to contact with fungicides and other seed-applied pesticides. When applied as a seed coat, adhesives are used such as sugars, natural and synthetic polymers (Deaker *et al*., 2004). There are environmental concerns in exploiting peat resources for which liquid inoculants are proposed to have less impact and represent a more suitable choice for the future (Ribeiro *et al*., 2013).

Unsuccessful inoculation is not always necessarily related to the inoculum quality nor inoculation method. Inoculation practices such as bad storage conditions and inappropriate manipulation also contribute to the bad quality of some inoculants, and deficient symbiotic results are observed in consequence.

Under some cropping systems, developing specific crop inoculants seems to have a greater potential in maximizing the BNF benefits. Selections from native strains with a specific crop approach for pea, lentils and vicias (*Vicia spp*.) in different regions of Spain showed enhanced

results and a potential to select strains that are crop specific and that also related to specific soil properties (Ruiz-Diez *et al*., 2012a; Ruiz-Diez *et al*., 2012b). Soil abiotic characteristics have demonstrated to have a substantial impact on the selection of strains for specific environments (Ruiz et al 2012b).

The most important challenges in optimizing the available inoculants and the inoculation practice rely on producing inoculants that are highly efficient with greater ranges of crops, or crop specific highly efficient according to the system, expanding to currently inoculated areas, and provide inoculants with stable efficiency under changing agri-environmental stresses (Santos *et al*., 2019).

2.2.5. Signaling in the legume - rhizobia symbiosis.

Legumes have the unique characteristic to interact with rhizobia in symbiotic relations. Rhizobia are soil-living gram-negative bacteria with genes for nodulation: *nod* and *rhi*, and for N fixation: *nif*, *fix*. The N fixing process, on which dinitrogen gas is converted to ammonia takes place in a structure called nodules, that are developed mostly in the roots of plants and on stems in some species (Sprent 2009). It is a structure that protects nitrogenase from inactivation in the presence of oxygen.

Legumes establish symbiotic relations with N fixing bacteria, exchanging a series of biochemical signaling events in order to allow the symbiont to enter the root. It is a host-specific interaction, in which rhizobia strains can infect a limited range of host plants. With the establishment of legume model systems in medicago (*Medicago trunculata*) and *Lotus japonicus*, major steps started to be done in understanding the nodulation signaling process (Young *et al*., 2003; Udvardi *et al*., 2005) as well as with the methods in functional genomics (proteomic, metabolomic and transcriptomics) (Stacey *et al*., 2006). Furthermore, the study of the evolution of microbes present on inoculants, as well as tracking lineages in complex multiply occupied interactions, will allow to better understand their ecology. Modelling this relationship will allow to predict the respond of symbiotic relations to environmental changes. This potentially has a great impact in maximizing yields and how nutrients are efficiently use in the agriculture system (Burghardt 2019).

Plants give the first signaling secreting flavonoids that are recognized by specific bacteria (Wang *et al*., 2012). Legumes secret different types of flavonoids, that will be responded by specific types of rhizobia, which had established the level of specificity between the host legume and the symbiotic bacteria (Wang *et al*., 2012). This induces nodulation genes (*nod* genes) encoding the enzymes that synthetize the specific nodulation signal: Nod factors in the bacteria (Freiberg *et al*., 1997; Dénarié *et al*., 1996). In the genus *Rhizobium*, genes *nod*, *noe* and *nol* are involve in the synthesis of Nod factors, and they cannot be expressed without receiving specific signals from the plant (Spaink, 2000) activating the root hair infection process and also letting the plant know more bacteria will infect (Geurts *et al*., 2005).

Differences in Nod factors among bacteria allow roots to recognize compatible symbionts (Lerouge *et al*., 1990), and the process will only happen if the plant has the matching Nod factor receptor for the bacteria; with the amount of Nod factors expressed by the bacteria also playing a part in the development of early nodulation structures (Downie 2010). Both quantity and quality of Nod factors are important for determining host specificity (Perret *et al*., 2000). Many different types of Nod factors create competition to even inhibit nodulation, given advantages to others to colonize (Walley *et al*., 2013). Few genes in the plant are involved in the control of the nodulation process and are recessive (Tsyganov *et al*., 1998). Nodulation in lentil occurs throughout root hair infection, where signalling is given by the plant, a hair is curled and bacteria entries forming an infection thread, which grows through cortex and branches systematically. It infects the symbiosomes (hosting membranes) and differentiates to its bacteroid form (Sprent *et al*., 2013; Doyle 2011).

First known event is the root hair curling, in which the bacteria get trap, and an infection thread is initiated. Throughout this structure, the bacteria are able to branch into the root cortex. A series of root cortical cell division is induced, and this division allows the beginning of the nodule primordium development (Stacey *et al*., 2006). Once the cells in the infection thread reach to the primordium, bacteria are released into the cell through endocytosis, where they differentiate into bacteroids, enclosed in a vacuolar structure: symbiosome. It is within this structure, called nodules, that bacteria can reduce atmospheric N_2 and provide the host a source ready to be used, NH_3 (Hirsch 1992).

All legume nodulating bacteria are phylogenetically organized according to 16S rDNA sequences (Young and Haukka 1996). The *nod* gene plasmids are known as pSym (Gonzalez *et al*., 1996) and their lateral transfer is involved in the evolution of symbiotic functions that explain inconsistencies found in *nod* gene phylogenetical trees (Wernegreen and Riley 1999). Although there is no complete congruence between 16S rDNA and *nod* genes, *nod* gene trees are better correlated with the range of host plants (Haukka *et al*., 1998), and have been vastly used for classification of strains (Ruiz *et al*., 2012b; Laguerre, *et al*., 2001). The 16S-23S rRNA intergenic spacer allows intraspecific differentiation, representing a good resource for occupancy tests (Ruiz-Diez *et al*., 2012b). This region is of importance to better understand relations among strains that have agronomical implications. In a study with strains isolated from Spanish soils, strains with equal 16S rRNA relatedness corresponded to their soil origin (Ruiz *et al*., 2012b). The study of *nod*C genes has been an important tool to understand the diversity within *Rhizobium leguminosarum* bv. *viciae* strains. Molecular markers base on these genes, have been demonstrated to be a good method to differentiate among strains from this species (Moschetti *et al*., 2005).

Hypernodulation has been studied in *Lotus japonicus* and is related to a shoot-localized ligand. HAR1, a protein controlling shoot apical meristem identity that could control nodule number (Krusell *et al*., 2002; Nisihmura *et al*., 2002). In pea the SYM29, and in medicago SUNN (HAR1 homologous protein) also required for the control of nodule number (Krusell *et al*., 2002; Schnabel *et al*., 2005). The communication throughout VATA1 proteins between shoot root, suggests plant development affects symbiosis, which in medicago is controlled by roots (Stacey *et al*., 2006).

Legumes develop the minimal number of nodules for optimal growth (Mortier *et al*., 2012). The control of effective fixation in the nodulation has been recently identified and determined by the *NFS1* and *NFS2* genes in medicago (Wang *et al*., 2018; Wang *et al*., 2017., Yang *et al*., 2017); and by the *APN1* on *Lotus japonicus* (Yamaya-Ito *et al*., 2018). There are determinate and indeterminate nodules (Sprent *et al*., 2013). Determinate nodules have a transient meristem with globose shape and the bacteroids population reach senescent at the same time (Rolfe and Gresshoff 1988). The indeterminate nodules have an elongated shape and the bacteroids are organized in differentiated zones: an active meristem, the infection zone, nitrogen-fixing and a

senescent zone (Vassey *et al*., 1990), that can turn into bifurcate, palmate or collaroid structures. Determinate nodules arise from the root inner cortex while indeterminate nodules expand their ramification in a zone behind the meristem and move to the cytoplasm of nodule cells (Gage and Margolin 2000). Nod factors (NF) are fundamental in the initiation of infection threads. Differences between determinate and indeterminate have been attributed to the *MtNFH1* NF mutation in medicago with different phenotypes of indeterminate nodules in the presence of reduced *MtNFH1* activity, with the strain *Sinorhizobium meliloti* 1021 (Cai *et al*., 2018). Nodule phenotypes are controlled by the legume plant and species that can produce both determinate and indeterminate types are uncommon (Fernandez-Lopez *et al*., 1998; Liu *et al*., 2014). *Lens* species produce both determinate and indeterminate nodules (Zahran, *et al*., 2013; Riah *et al*., 2014).

2.2.6. Specificity of the legume – rhizobia relationship.

Legume plants evolved to efficiently interact with very few symbiotic species. This level of specificity is largely determined by the NF receptors in the host plant. This is key for the establishment of effective symbiotic relations (Bisseling and Geurts 2020). The specificity in the medicago – *Sinorhizobium* phylogenetical relatedness seems to be associated with the geographical origin of bacteria, and these impacts the distribution of plant species and how they colonize new areas (Bena *et al*., 2005). The genes *NFS1* and *NFS2* in medicago have been recently identified to restrict effective fixation in the nodulation with specific strains (Wang *et al*., 2018; Wang *et al*., 2017., Yang *et al*., 2017), by the *APN1* gene in *Lotus japonicus* (Yamaya-Ito *et al*., 2018); and the *Rj2* in soybean (Yang *et al*., 2010).

It is also known that the plant plays a key role in selecting for better symbionts for its capacity to control the oxygen supply to those who are fixing less N when it is infected with a diverse set of bacteria (Denison and Kiers 2004). Soybeans have also demonstrated to select for specific symbiotic partners during growth, when inoculated with multiple strains (Sugiyama *et al*., 2015; Santos *et al*., 1999).

The role of specificity at the level cultivar × strain has proven to be important on the effectiveness of this relation in a number of legume crops. Both the strain and legume cultivar are determinant on the N fixation effectiveness of this symbiotic relation at different levels. In some cases, can be very specific as in pea (Yang *et al*., 2017), common bean (Gunnabo *et al*., 2019; Valberde and Ottabong 1997), medicago (Bena *et al*., 2005), soybean (Ramongolalaina *et*

al., 2018; Sugiyama *et al*., 2015) and lentil (Abi-Ghanem *et al*., 2011). In other cases, can have a moderate specificity as is the case of tepary bean (*Phaseolus acutifolius*) (Somasegaran and Hoben 1991). Very promiscuous association have been identified mostly in tropical legumes such as *Arachis* species (Somasegaran and Hoben 1994).

2.2.7. The BNF process in lentils.

Rhizobium is one of the six genera of bacteria that are known to nodulate legumes. *Rhizobium leguminosarum* bv. *viciae* is the symbiont of lentils, but it also forms associations with other *Vicieae* (*Vicia*, *Pisum*, *Lathurys*). There are several *Rhizobium* species known to nodulate lentil that were selected in specific areas in symbiosis with lentils over the course of domestication. *Rhizobium leguminosarum* bv. *viciae* is the symbiont on lentils in the Middle East and Europe (Harun-or-Rashid., *et al*., 2014), in Bangladesh three other lineages: *R. bangladeshense*, *R. lentis*, *R. binae* of the symbiont very well separated originated with the group of lentils *grex pilosae* (Harun-or Rashid *et al*., 2012). *Rhizobium laguerreae* is the main symbiont of cultivated lentil in Morocco (Taha *et al*., 2018). There are other species nodulating lentil in New Zealand yet unnamed (Gai *et al*., 2021).

Lentils are the sixth on their contributions of N fixation after soybean, lupin, pea, faba bean and common bean. Its contributions are compared to those made by chickpea and common bean with about 80 kg N/ha from the whole plant (Unkovich and Pate 2000). The 3.8 million ha of lentils accounted for 0.7 Tg of N/year until 2007 (McNeil and Materne 2007) of the 90 Tg fixed by legumes in the world annually. The world's average in lentil for the amount of N fixed in farmer's fields is 72 kg shoot N ha^{-1}, with South Asia with the lowest value of 53, and Oceania the highest of 90 (McNeil and Materne 2007). These values of kg shoot N ha^{-1} range broadly from 4 to 152 (Peoples *et al*., 2009). %Ndfa in lentil is 63% and ranges from 28-87% (Unkovich and Pate 2000). Estimates of the total amount of N fixed by lentils range from 0-192 kgN/ha, with an average of 80 kg N/ha. Net contributions to soils can vary from 25-75% depending on the amount of N removed relative to the amount of N fixed (Salvagiotti *et al*., 2008, Walley *et al*., 2007).

Studies have been conducted to determine the effects of plant genotype and strains and their impact in their symbiotic relation. Lentil genotypes are stablished to have an effect in nodulation characteristics including nodule weight, nodule number and amounts of N fixed (Hafeez *et al*.,

2000). Several studies have demonstrated the variation among strains (Hafeez *et al*., 2000; Bremer *et al*., 1990; Martinez-Romero 2003). The relation between *Rhizobium* and lentils have been studied on the areas where lentil is grown under controlled and field conditions. Studies in Pakistan under low N conditions, with *Rhizobium leguminosarum* and several lentil cultivars, showed differences for the number of nodules, dry weight of nodules, biomass yield, total N yield and %Ndfa and also suggested specificity at the cultivar × strain level (Haffeez *et al*., 2000).

The effects of the cultivar were tested with 5 varieties of lentils inoculated with 18 strains of *Rhizobium leguminosarum* bv. *viciae* under greenhouse conditions in Pullman WA, using N-free potting mix, N-free nutrient solution and a low level of N fertilizer enriched with ^{15}N. Variation among varieties was found on the proportion of Ndfa. Legume varieties had more influence on the measured traits than the strains, suggesting the possibility to breed for increasing N fixation (Abi-Ghanem *et al*., 2011). The commercial strains evaluated in this study represented interactions with *Rhizobium leguminosarum* bv. *viciae* and don't represent the diversity of isolates known to nodulate lentil nowadays. Different cultivars were also studied under rainfed conditions in Syria, where the cultivar Horani adopted by farmers, showed the best results, which can be attributed to the adaptation with local strains over the years, and better performance nodulation characteristics were associated to small-seeded lentil types (Kurdali *et al*., 1997).The effectiveness of 229 strains, isolated from inoculated lentil plants grown in Turkey, Syria Jordan and Egypt, with the variety ILL 16 were tested in a hydroponic system, finding desirable nodulation with 21% of the strains, corresponding mainly to strains from Jordan and Turkey (Moawad and Beck 1991).

The amounts of N for subsequent crops are about 23-45 kg N/ha. (Kessel and Hartley 2000; McNeil and Materne 2007). Crop rotation with lentils have demonstrated to enhance yields in other crops. This increasing in yield as well as higher protein content of the cereal or oilseed grown in rotation, was a response to favorable BNF but also to agronomic management (Gan *et al*., 2003; Miller *et al*., 2003). Average estimates of the N fixed by lentil in 80 separate sites determined that for lentils to make an actual contribution, it needs to obtain 48% of Ndfa (Walley *et al*., 2007). Considering that this value is in average 63% and ranges from 28-87% (Unkovich and Pate 2000), lentils will likely contribute a positive N balance.

2.3. Techniques and parameters for quantifying BNF

Increasing BNF ability requires substantial research to improve the main components (legume and symbiont) and then have them applied to the cropping system. This is possible by conducting experiments that identify their effects on N fixation, and effectivity on cropping systems, which requires accurate and reliable quantifications. These are linked to the degree to which scientists have access to appropriate methodologies for measurement of the components (Unkovich *et al*., 2008).

There is no right or wrong way to measure N fixation since all known techniques have a degree of limitations (Azam and Farooq 2003), which makes it important to evaluate several parameters and complementary measurements for a more accurate interpretation of the data. Choosing the most sophisticated or expensive methods will not necessarily give more accurate nor useful information. Evaluations should rather focus on evaluating more parameters rather than the most accurate N fixation estimates. Regardless of the technique used, the quantification of N fixation requires estimating plant dry matter and concentration of N in the dry matter. Also, the presence of nodulation (number of nodules, color, nodule biomass, appearance), separate the proportion of the Ndfa (using N difference, ^{15}N natural abundance, ureides) and a measure of nodule activity (Unkovich *et al*., 2008).

The most important is to clearly state the objective and use the method that will respond the hypothesis questions. For example, if the objective is to authenticate a strain, inoculation, and observation of nodulation with desirable characteristics in the legume will be sufficient, and no estimation of N fixation nor any other method needs to be used. Some of the general goals in the study of the BNF include determine plant genotype's ability to nodulate and fix N, effectiveness of strains with the plant genotypes of interest, total amounts of N fixed, comparations among individuals and effects of the environment and cropping conditions on the success of the fixing process. Additionally, many BNF studies have focused on the accurate measure of above-ground N fixation, ignoring the role that N accumulation in roots, that has been demonstrated to be underestimated (McNeil *at al*., 2008; Khan *et al*., 2002). In lentils, roots account for about 20-25% of the total fixed N in the plant (Unkovich and Pate 2000).

Implementing the right methodology as well as establishing measurements of efficiency, will better assess results that have an impact in the system that is attempted to improve. In general,

the simplest and least expensive method that serves the objectives should be chosen (Azam and Farooq 2003).

When measuring the effectiveness of strains, comparisons with standard well-established strains and with a legume receiving adequate N should be implemented. Numerous groups of strains can be testes under controlled conditions, but performance of strains has to be evaluated under field conditions. Better expression of N fixation it is observed under low mineral soil N content (Somasegaran and Hoben 1991). It is required that the strain is first authenticated with the legume to determine the strains' ability to infect. After this, the strains effectiveness has to be determined by its ability to fix N with the host legume. It is crucial to determine the strains' ability to persist in the local field thorough multiple seasons, because this ability cannot be predicted without field experimentation (Howieson *et al*., 2016). Optimum results of inoculation have been compromised in legume programs for the lack of availability of strains adapted to field conditions (Beaker *et al*., 2004). Introducing strains to a new soil represent a very complex process that has to take into account the effects the strain will have in the soil microbiota as well as the effects of the soil in the strain's performance. This has been extensively studied and for the very specific conditions on which the strain will be in symbiosis with the crop. Some of the common studied conditions include the performance of strains when there is a stablished *Rhizobium* population both low and with high counts, abiotic stresses such as acidity, alkalinity and low clay, and persistence thorough rotations (Loi *et al*., 2005; Howieson and Ballard 2004; Graham 1992; Thies *et al*., 1991, Helyar and Porter 1989).

To compare between genotypes, *Rhizobium* strains or treatments, different techniques can be used, and availability of equipment, supplies, accuracy required, and all management practices involve, should be taken in consideration before deciding, but combinations of different methods have given the most accurate results (Unkovich *et al*., 2008).

The N difference method compares the total N fixed by the inoculated plant against a non-fixing, or non-inoculated plant. It allows to separate the estimated total amount of N fixed from the mineral N obtained from the soil or growing media. It provides a low-cost method and can be very accurate when is conducted under low N conditions with the appropriate reference plant (Mapfumo *et al*., 1999). The major limitation associated with estimating N fixed with this

method, is the use of other plant species as a referent plant with different abilities to use soil N, in most cases cereals (Chalk 1998).

The N balance method provides the net increase in N fixed by the plant in the system. The accurate estimation requires either the absence of other N sources for the plant or quantification of mineral N accessed by the plant for which fixing ability is attempted to be estimated. The potential limitations with this method are associated with difficulties to estimate any present N in the media where the plant is growing (Chalk 1998). The N difference and the N balance methods are the simplest available for N fixation quantification. Other isotopic methods are available for studying the BNF process, and aspects to consider the chosen method are described in table 2.1.

Table 2.1. Characteristics of methods to quantify biological nitrogen fixation. "×" used to represent the method has the characteristic. Based on Unkovich *et al*., 2008.

Characteristics	**Non-isotopic methods**			**Isotopic methods**		
	N balance	**N difference**	**Ureide**	**C_2H_2 reduction**	**^{15}N natural abundance**	**^{15}N enrichment**
Time integrated	×	×			×	×
Reference plant		×			×	×[c]
Non-destructive			×[a]		×[a]	
%Ndfa	×	×[d]	×		×	×
Quantifies N yield	×	×	×		×	×
Laboratory			×	×		×
Glasshouse	×	×	×	×	×	×
Field	×	×	×		×	×
Precision	low	low-medium	good	low	Low-good[b]	Medium-good

[a]If only %Ndfa is required. [b]Depending on natural enrichment of soil. [c]Not when cultivated in N-free media. [d]Can be calculated indirectly.

used as a rapid, non-destructive and accurate technique to measure leaf chlorophyll concentration (Ling *et al*., 2011). Root biomass and N content are also valuable to estimate, as well as root architectural characteristics. When available direct estimates of root N content can give more accurate estimates for the genotype N fixing ability (McNeil *at al*., 2008). Architectural

N fixing related parameters should be phenotype at flowering. Nodulation is evaluated by phenotyping the nodules in roots. Some of the parameters are number of nodules, mass, nodule color, distribution in the root system and speed of nodulation. Most evaluation systems have developed scales based on rapid counts or approximate estimations of the number of nodules especially when conducting phenotyping in the field (Corbin *et al.*, 1977).

When observations of the N fixing activity, seed production and more variables are taking into consideration, greater number of nodules or nodule mass are not necessarily good indicators of higher BNF ability (Yang *et al.*, 2017). Efficiency must be a rate of mg of N fixed/gram of nodule weight (Somasegaran and Hoben 1991).

Plant measures need to be taken including biomass and estimates of the N accumulated. N accumulated can be estimated directly or indirectly such as SPAD (Rowe and Cadisch 2002). Characteristics can be obtained precisely with a computer-based analysis and root measuring software such as WinRhizo© and provide accurate information (Burridge *et al.*, 2016), but soil coring and phenotypic distributions have also given relevant information (do Rosario *et al.*, 2000).

The total amount of N fixed will require dry biomass, N concentration and total N accumulated in the plant. Results are measured in mg N/plant when evaluating single plants or kg N/ha in the field. When estimating N root concentration, a 1.4 factor is suggested for the estimations of N accumulation in lentil (Unkovich *et al.*, 2008). The most important measure to determine the benefits of N fixation is seed yield (Unkovich *et al.*, 2008).

Seed yield and comparison to N fertilized plants should be conducted. Protein and protein yield are also important measures for understanding translocation of N to seed products (Zimmer *et al.*, 2016). Different systems are used for the evaluation of symbiotic relations under controlled conditions. Some of the most used apparatus to screen for BNF are described in table 2.2.

Table 2.2. Apparatus for screening rhizobia for nodulation and effectiveness with a guide to their advantages and disadvantages, from Howieson *et al*., 2016.

Assembly and growth medium	Advantages	Disadvantages
Glass tube with vermiculate, sand or agar support medium.	Can be sterilized. Control of hygiene. Space efficient.	Not all legumes fix N_2 in polycarbonate tubes.
Plastic growth pouches with liquid medium.	Space efficient. Can visualize root system.	Can be expensive. Difficult control of airborne contaminants.
Leonard jars containing sand or vermiculite, with nutrient solution	Can screen large-seeded legumes (e.g. soybean and faba bean).	Time and labor intensive.
Enclosed polycarbonate 'O Hara' vials.	Easy to set-up. Can be sterilised as a unit. Recyclable and space efficient.	Not all legumes fix N in enclosed vials.
Sand with nutrient solution in plastic pots, with surface-applied beads.	Can screen large or small legumes, with several species per vessel. Sand better reflects field conditions, allows deep drainage and is cheap to procure.	Requires adequate space and a clean greenhouse.
Intact soil cores.	Can mimic soil physical and chemical conditions.	Time and labor intensive in core collection.
Hydroponic solution	Can closely monitor nutrient flows. Space efficient and roots easily examined.	Difficult to control contamination. Limited rhizosphere development.

2.4. Breeding for enhancing BNF

2.4.1. Role of wild reservoirs in BNF.

Wild species and species related to our crops represent diversity is needed to be re-introduce for traits needed to face current and future challenges in agriculture. Rhizobia populations from wild legumes have become an important focus of study, because of their diversity, promiscuity and ability to survive under arid conditions, and their ability to colonize wild and domesticated plants equally (Zahran 2001). Indigenous N fixing bacteria has proven to not only improve symbiotic processes in main plant species but also impacted soil quality and fertility (Requena *et al*., 2004), and are also a source of desirable genes for improving symbiotic performance (Appunu and Dhar 2008a).

Both strains from native ranges as well as wild legumes related to crops have proven to contribute the BNF process.

In white clover (*Trifolium* spp.), native strains have proven to have similar and greater nodule occupancy compared to commercial strains and 5 times greater in the infection of stolon roots. Same strains have proven to be better to those selected 10 years before because of environmental changes (Irisarri *et al*., 2019). Interspecific hybrids between (*Phaseolus vulgaris* × *Phaseolus acutifolius*) had enhanced BNF characteristics compared to their parents, when inoculated with a group of *Rhizobium* (TAL 182, TAL1383, TAL 1797) and *Bradyrhizobium* (TAL 336, TAL 644, TAL 648) strains. Most of the hybrids stablished symbiosis with *Bradyrhizobium* strains, which are in general a more efficient symbiont than *Rhizobium* (Somasegaran and Hoben 1991).

Chickpeas inoculated with commercial and native strains of *Mesorhizobium ciceri* in different chickpea cultivars, showed the highest contributions in N fixed from the wild isolates (Abi-Ghanem *et al*., 2012). Also, differences were observed on the cultivar effect, suggesting efforts for improving BNF should be focusing on varietal breeding and strain selection to increase agricultural N fixation (Abi-Ghanem *et al*., 2012). In soybean, a study with 31 cultivated and 17 wild accessions, suggests that improvement in BNF could be part of the domestication process for this crop. There is exceptionally high accumulation of poly-B-hydroxybutyrate bodies in bacteroid of the nodules of wilds, suggesting carbon/nitrogen balance is not good for fixation (Munoz *et al*., 2016). The role of wild reservoirs of *Lens* on the BNF in the lentil crop is yet to be determined.

Modern varieties are usually selected under high fertility conditions, where all necessary nutrition is provided in available forms from synthetic fertilizers, making it unnecessary for the plant to establish effective interactions with symbiotic soil microorganisms, contributing to the loss of genes that control these traits (Wissuwa *et al*., 2009). It is possible that such alleles compromise yield under high fertility, given that nutrient acquisition throughout microbial interactions is an energy-costly process for the plant (Lambers *et al*., 2006). The capacity of a plant genotype to efficiently interact with beneficial microbial seems to be an inherited trait (Rengel 2002). Furthermore, most of the important cultivated plant species have not surpassed the biological limit on nitrogen use observed for wild species (Cipriotti 2016).

Methods in lentil breeding are those used in other self-pollinated crops: combinations of bulk, pedigree, and single-seed descent. The inclusion of unadopted germplasm is conducted mostly using multi-parental crosses (Slinkard, *et al*., 2002; Erskine *et al*., 1990; Kahraman *et al*., 2004).

Interspecific hybridization has the capacity to generate phenotypic novelty, both to understand the genetic basis of traits with agronomic value, and as the vehicle for the introgression of traits no longer present in the cultivated pool. For the BNF this approach has been successful in common bean (Somasegaran and Hoben 1991), a legume with lower capacity to fix N compared to most of the crop legume species. Contributions from the related species, *Phaseolus acutifolius*, through interspecific hybridization have been demonstrated, resulting in common bean germplasm with a higher capacity to fix N and ability to establish symbiotic relations with *Bradyrhizobium* species.

Improving the legume-*Rhizobium* relationship not only involves improving the main components (legume and rhizobia), but also doing it under multiple agro-environmental factors that affects this relationship such as the microbial populations in the soil, structure and nutritional composition of the soil, acidity, salinity and abiotic factors that have been directly measured for specific cropping systems (Zarei *et al*., 2006; Singh *et al*., 2005; Kessel and Hartley 2000; Ruiz-Diez *et al*., 2012). Basically, this relationship will be affected by anything that affects photosynthesis, which can lead to low heritability values and genetic gains (Graham *et al*., 2004). This makes indirect selection methods an attractive and more efficiently approach for a breeding program.

Using linked markers for indirect selection for rhizosphere related traits of interest would be ideal/helpful because of the difficulties of evaluating and selecting for them directly. A quantitative trait loci (QTL) is a region in the genome associated with a particular phenotype. A number of QTL have been identified for symbiotic traits in legume systems. The first QTL responsible for symbiotic traits were reported in the model legume *Lotus japonicus* for nodule number and nodule weight and other related traits (Tominaga *et al.,* 2012). In common bean QTL have been associated with Ndfa (Kamfwa *et al*., 2019) and in cowpea (*Vigna unguiculata*) with nodule fresh weight (Ohlson *et al*., 2018). QTL have been also found for total nodule fresh weight in interspecific populations of soybean crossed with its wild relative (*Glycine soja*) inoculated with *Bradyrhizobium*.

2.4.2. Molecular breeding tools

Marker assisted selection (MAS) is largely used in breeding programs yet are poorly utilized for selecting for enhanced BNF in legume breeding programs. Molecular markers of DNA

polymorphism are one of the most important goals in plant breeding to detect genomic variation. The single nucleotide polymorphism SNP (SNP) is the most abundant type of genomic variation in nature (sequence-based). The detection of high-throughput variation across the genome is possible with the current next-generation sequencing techniques. SNP detection allows the use of polymerase chain reaction (PCR) based markers such as Kompetitive allele specific PCR (KASP). There are several types of markers and choosing a suitable type for the breeding program depends not only on the market type, but also on the resources and using practicality in the breeding program.

Quantitative traits are measurable continuous phenotypes that depend on the action of multiple genes and are also influenced by the environment. The quantitative trait loci (QTL) are the causative regions in the genome associated with this variation. QTL mapping and association mapping are used for the detection of this regions. QTL mapping uses a set of markers and the phenotypic variation expressed by a bi-parental population. With association mapping the variance among unrelated individuals can be identified by linkage disequilibrium. QTL mapping allows the detection of regions containing putative genes of importance in breeding selection. Given that the parental lines are the source of variation in a mapping population, significant QTL could be restricted to that specific population. The statistical significance of QTL mapping is also affected by the quality of phenotypic data, population size and inbreeding generation.

QTL mapping research started to be conducted in lentil when PCR-based markers became available. Most of the earlier studies have focused on disease resistance (Tullu, *et al*., 2006; Saha *et al*., 2009; Chowdhury *et al*., 2001), and agronomical traits (Kahraman., *et al*., 2004). Recently, genome-wide high resolution linkage analysis (Jain *et al*., 2013; Sharpe *et al*., 2013), and next generation sequence technology (Kumar *et al*., 2015) allowed the mapping of complex traits in lentil, and the acceleration of marker-assisted breeding (Singh *et al*., 2017; Kumar *et al*., 2015). The lentil reference genome became available (CDC Redberry), and wild genomes of *Lens* species are becoming available and will facilitate marker-assisted breeding on interspecific.

2.5. Terminology

Table 2.3 describes some key terminology that will be used throughout this thesis and is presented to facilitate reading it and clarify their specific uses in the context of this study.

Table 2.3. Overview of the terminology used throughout the thesis.

Term	Acronym	Description
Nitrogen	N	Elemental nitrogen (e.g.: mineral N from the soil or, total N accumulated).
Dinitrogen	N_2	Gaseous dinitrogen. Used when referring to N obtained by the plant from fixation.
Added nitrogen	+N	Chapters 3 and 5. Nitrogen added in the form of chemical fertilizer.
N_2 fixed, N fixed	-	Nitrogen obtained by the plant via nitrogen fixation
N accumulated		Nitrogen accumulated from mineral sources.
Rhizobium	R	One of the six main genus known to stablish symbiotic relations with legumes.
Rhizobia	-	Whole present population of N_2 fixing bacteria that could belong to more than one genus and or species.
Strains	-	N_2 fixing bacteria of known taxa.
Isolates	-	N_2 fixing bacteria of unknown taxa.
Control	C	Chapter 3: non-inoculated, no +N, with low mineral N plant unit used to estimate N fixation with the N difference method, and absence of rhizobia different from the inoculated strain. Chapter 4: non-inoculated treatment with the presence of soil-established rhizobia.
Number of nodules	TNN, NN1, NN2 or NN	Chapter 4: NN1 and NN2: nodules established at specific depths in the root system. TNN= NN1+ NN2. Chapters 3, 5 and 6: NN: total number of nodules in one plant.
Site-year	-	When unequal number of experiments are conducted in different sites each year.
species	(*species*)	All *Rhizobium* strains and *Lens* accessions are presented with their respective species. The abbreviated form is presented to facilitate reading. e.g.: IG 72643 (*L. orientalis*) and NZLR-24 (*Rlv*).
Accession	-	Uniquely identified plant source wild or cultivated.
Cultivar	-	Plant variety produced by selective breeding.
Genotype	-	When referring to the specific set of genes conferring a characteristic to a specific accession.
Inbred line	-	Produced by inbreeding. When referring to an individual member of the interspecific populations (Chapter 6).

Prologue to Chapter 3

This chapter is about the effect diverse *Lens* species have in the relationship with rhizobia, based on the interaction with the commercial strain BASF 1435 (*Rhizobium leguminosarum* bv. *viciae*).

This chapter will be submitted to Crop Science. Paper and co-author contributions will be as follow:
Vargas, A., Gorim, L.Y., Vandenberg, A. and K.E. Bett. 2021. Differential nitrogen fixation ability among *Lens* species. Crop Science *in preparation.*

Author contributions:
Vargas, A.: responsible for design, execution, analysis of experiments and preparation of manuscript with input from Bett, K.E.
Gorim, L.Y.: phenotyping and will review and edit the manuscript.
Vandenberg, A.: development of initial concepts for the experiments and will review and edit the manuscript.
Bett, K.E.: development of initial concepts for the experiments and funding acquisition. Supervised Vargas, A., revised and edited the manuscript.

3. DIFFERENTIAL NITROGEN FIXATION ABILITY AMONG *LENS* SPECIES

3.1. INTRODUCTION

Wild relatives and related species are the greatest source of genetic diversity for crop breeding, representing a key element for adaptation to changing conditions and food security (Global Crop Trust 2019). The inclusion of such diversity through interspecific hybridization, has resulted in significant progress in the breeding for resistance to biotic and abiotic factors for the most important grain legume crops such as soybean (*Glycine max*), peanut (*Arachys hypogaea*), chickpea (*Cicer arietinum*), lentil (*Lens culinaris*) and common bean (*Phaseolus vulgaris*) (Muñoz *et al*., 2017).

Modern varieties are usually selected under high fertility conditions, where all necessary nutrition is provided in available forms from synthetic fertilizers, making it unnecessary to establish symbiotic relations, contributing to the loss of genes that control these traits (Wissuwa *et al*., 2009). Breeding objectives for lentil have been mainly focused on yield and specific seed characteristics, increasing the production but also narrowing the genetic variability (Erskine *et al*., 1998) and consequently, reducing the levels of resistance to biotic and abiotic factors.

There are six wild relative species in the genus *Lens* (Wong *et al*., 2015) and several have been used for introducing genetic variation into the domesticated gene pool. These species have been identified as good sources of disease resistance such as anthracnose, ascochyta blight and stemphylium blight (Vail *et al*., 2012; Podder *et al*., 2012; Gupta and Sharma 2006). Differential root traits among genotypes, root distribution and drought tolerance mechanisms have been also reported for wild *Lens* accessions (Gorim and Vandenberg 2017).

The biological nitrogen fixation process (BNF) contributes about a third of the N that is used in agroecosystems annually (Herridge *et al*., 2008). This is converted into protein available for human consumption as fresh vegetables and dried grains but also leaves below ground contributions to subsequent crops (Aslam *et al*., 2003). The proportion of N derived from the atmosphere (Ndfa) in lentil is around 65%, but a broad range of estimates have been reported from 9-97%, with values of kg shoot N ha^{-1} from 4 to 152 (Peoples *et al*., 2009). In Canada, values of the Ndfa vary from 0-87%, with amounts of 0-192 of total N fixed in kg/ha (Walley *et al*., 2007).

The original symbionts of lentil are strains from the species *Rhizobium leguminosarum*, but 3 more lineages of *Rhizobium* are associated with lentils in Bangladesh (Harun-or Rashid *et al*., 2014), *R. laguerreae* is the symbiont in Morocco (Taha *et al*., 2018), and several additional species are being characterized (Gai *et al*., 2021).

The effect of lentil genotype has been tested under controlled conditions with *Rhizobium leguminosarum* bv. *viciae* with significant variation noted among varieties for the proportion of Ndfa, suggesting the importance of breeding for increasing N fixation (Abi-Ghanem *et al*., 2011). In common bean, the contributions from crosses with the related species tepary bean (*Phaseolus acutifolius*) through interspecific hybridization have resulted in germplasm with higher capacity to fix N (Somasegaran and Hoben 1991). In soybean, higher N fixing ability is suggested to have been gained during the domestication and breeding processes, based on a study of a group of wild and cultivated genotypes, as well as a recombinant inbred line population (Muñoz *et al*., 2016).

The improvement of rhizobia-legume symbiosis is the most important route for increasing efficient use of N in cropping systems. It is expected that contributions can be made to the N fixing ability of lentil from the available diversity in cultivated lentils or its wild relatives. This study was designed to identify species and genotypes from a broad set of accessions from the genus *Lens* that could be useful in breeding for better BNF ability.

3.2. MATERIALS AND METHODS

3.2.1. Accessions and *Rhizobium* strain

The 36 accessions, representing a cross section of all *Lens* species, that were tested are listed in table 3.1. The inoculant used for the experiment was the strain BASF 1435 Nodulator XL® *Rhizobium leguminosarum* bv. *viciae* (*Rlv*), a commercial inoculum currently used by farmers in Saskatchewan, Canada. This strain would constitute part of the resident population of rhizobia in pulse-growing areas of the Northern Great Plains as it has been used for at least 2 two decades.

3.2.2. Growing conditions and inoculum

The experiments were conducted in the greenhouse facilities at the U of S during 2017-2019. Two experiments were planted, in May and August 2017, for evaluation at flowering.

Table 3.1. Accession, species, gene pool and origin of 36 *Lens* species accessions studied for their N fixing ability with the strain BASF 1435 Nodulator XL® (*Rhizobium leguminosarim* bv. *viciae*).

Accession	M[a]	Species	Gene Pool	Country of Origin/Source Institute
CDC Maxim[c]	×	*L. culinaris*	Primary	Canada
CDC Redberry		*L. culinaris*	Primary	Canada
CDC Robin		*L. culinaris*	Primary	Canada
CDC Milestone		*L. culinaris*	Primary	Canada
CDC Asterix		*L. culinaris*	Primary	Canada
CDC KR-1	×	*L. culinaris*	Primary	Canada
CDC Greenstar	×	*L. culinaris*	Primary	Canada
CDC QG-4		*L. culinaris*	Primary	Canada
Eston		*L. culinaris*	Primary	Canada
VIR-421		*L. culinaris*	Primary	Russia
Lupa	×	*L. culinaris*	Primary	Spain
ILL 7502		*L. culinaris*	Primary	ICARDA[d]
ILL 1704		*L. culinaris*	Primary	ICARDA
ILL 8006		*L. culinaris*	Primary	ICARDA
Indianhead	×	*L. culinaris*	Primary	Canada
BGE 016880	×	*L. orientalis*	Primary	Israel
IG 72529		*L. orientalis*	Primary	Turkey
IG 72611		*L. orientalis*	Primary	Turkey
IG 72622		*L. orientalis*	Primary	Turkey
IG 72643	×	*L. orientalis*	Primary	Syria
IG 72672		*L. orientalis*	Primary	Turkey
PI 572376	×	*L. orientalis*	Secondary	Turkey
IG 72613		*L. tomentosus*	Secondary	Turkey
IG 72614		*L. tomentosus*	Secondary	Turkey
IG 72805		*L. tomentosus*	Secondary	Turkey
PI 572390	×	*L. tomentosus*	Secondary	Turkey
IG 72543		*L. odemensis*	Secondary	Palestine
IG 72623	×	*L. odemensis*	Secondary	Turkey
IG 72760	×	*L. odemensis*	Secondary	Syria
IG 110810	×	*L. lamottei*	Secondary	Spain
IG 110813		*L. lamottei*	Secondary	Spain
IG 72537		*L. lamottei*	Secondary	France
IG 72815		*L. ervoides*	Tertiary	Turkey
L01-827A	×	*L. ervoides*	Tertiary	Unknown[b]
LR59-81[e]	×	*L. culinaris* × *L. ervoides*	interspecific	Canada[b]

Table 3.1. Continued.

Accession	M[a]	Species	Gene Pool	Country of Origin/Source Institute
IG 116024		*L. nigricans*	Quaternary	Turkey

[a]M: also evaluated at maturity, [b]Fiala *et al*., 2009. [c]CDC: Crop Development Centre, University of Saskatchewan SK Canada. [d]ICARDA: International Center for Agricultural Research in the Dry Areas. [e]LR59-81: interspecific line (Eston × L01-827A), CDC, University of Saskatchewan, Canada. White: cultivated and grey: wild accessions.

Two subsequent experiments, with a subset of 14 accessions, were planted in February and October 2019 for evaluation at maturity. Seeds were disinfected, scarified and pre-germinated in soft agar (6% w/v) 48-72 hours before planting. For disinfection, seeds were surface sterilized with 70% ethanol (v/v) for 30 seconds, followed by 5% bleach (v/v) for 2 minutes, and washed with running distilled water. Scarification was conducted to ensure germination of the wild accessions and carried out manually by nicking the seed coat with a razor blade before plating, about 30 seeds per accession.

A low-N growing mix composed of Sunshine® #3 (Sungro Horticulture, MA USA) and sand (1:1 v/v), pasteurized for 72 hours at 70℃, was used to ensure the absence of rhizobia and other microbial populations. N-free nutrient solution (Somasegaran and Hoben 1994) was applied to the growing media 24 hours before planting the germinated seeds in polyvinyl chloride (PVC) cylinders (11 cm diameter × 40 cm depth). The chemical N treatment consisted of a split application of CH_4N_2O (Urea), applying 25% along with the nutrient solution and 75% two weeks later (219 mg of N per cylinder total). All treatments had optimal fertility for all the other elements to ensure full N fixation potential. Amounts of nutrients per cylinder were: 529 mg of P, 402 mg of K, 53 mg of Mg, 49.5 mg of Zn, 53 mg of Ca, 1.95 mg of Mo, 1.85 mg Mn, 2.15 mg of Cu, 6.7 mg Fe, and 1.8 mg of Co. Sources of fertilizers were: KH_2PO_4, triple superphosphate (TSP), $MgSO_4.7H_2O$, $CaCl_2$, $ZnSO_4.7H_20$, $Mo_7O_{24}.H_2O$, $MnSO_4.H_2O$, $CuSO_4.5H_2O$, H_3BO_3, $FeC_6H_5O_7.3H_2O$ and $CoSO_4.7H_2O$. The same amount of water was applied to each cylinder: from 0-7 DAS 200 ml total, from 8-14 days 70 ml daily, from 14 until flowering 150 ml daily. For the experiments evaluated until maturity, 250 ml of water were added daily from flowering to maturity.

Pure *Rhizobium* culture grown on yeast mannitol agar (YMA, Sigma Aldrich #Y3252) (Table A, Appendix A) was transferred to yeast mannitol broth (YMB, Sigma Aldrich #Y3377). The liquid

culture was agitated in a shaker (Orbital, Thermo Scientific, Waltham MA) for 48 hours at 26℃ and 180 rpm. Liquid inoculum was standardized to 3×10^9 cells/ml and inoculation was conducted 24 hours after transferring the germinated seeds to the growing medium by applying 1 ml of liquid inoculum to each cylinder.

3.2.3. Experimental Design and Statistical Analysis

Treatments were arranged in a split-plot design to prevent infection of rhizobia in the control (C) and added N treatments (+N), with each cylinder representing an experimental unit. Treatments were the main plots: R: *Rhizobium,* no added N; C: no *Rhizobium,* no added N; +N: added N, no *Rhizobium*. The 36 lentil accessions were randomized to the subplots in four blocks, for a total of 432 experimental units and the whole experiment was repeated twice. The same treatments, distribution and replicates were used for subsequent experiments evaluated at maturity, with 14 selected accessions (Table 3.1), for a total of 168 experimental units conducted twice.

For the statistical analysis, an ANOVA test was conducted with main plots and accessions considered as fixed and blocks as random to test for significance of effects. Means were separated with the least significant difference test (LSD; $P\leq0.05$) using the statistical package SAS (SAS Institute, 2015). A Pearson correlation matrix was also generated among all measured parameters in SAS. It was conducted with the 14 accessions evaluated at maturity to establish relations among parameters evaluated at flowering and maturity. Control (C) results were presented only for the parameters related to N fixed.

3.2.4. Phenotyping

3.2.4.1. At flowering

Because of the variation in phenological cycles, plants were evaluated as they flowered (first open flower), to ensure they were all the same stage when the measurements were taken. Days to flower (DTF) were recorded. Right before sampling plants, leaf chlorophyll concentration was determined with a SPAD-502® meter (Spectrum Technologies, Aurora, IL). Shoots were separated from roots with scissors and dried at 70℃ for 72 hours to determine shoot dry weight (SDW-g). A shoot subsample was ground and passed through a 2 mm mesh screen and N concentration in shoots was determined on 100 mg samples using a LECO analyzer (Leco FP628, Leco Corporation, St. Joseph, MI). N accumulation was estimated as a product of N concentration in shoots and SDW. These values were used to determine the N fixed by using the

N difference method (Equation 3.1; Unkovich *et al*., 2008). Each accession was included in the control and N fixation was estimated using the matching genotype as the reference plant. Roots of plants in the controls were examined to ensure absence of nodulation.

N_2 fixed (N difference method) =
[N yield inoculated plant (N/100*SDW) - N yield control plant (N/100*SDW)]

Equation (3.1)

Roots were washed using a 5 mm screen separating all growing media from the root while keeping the entire root system. Washed roots were placed in Ziploc® bags in a fridge at 6 ˚C until they were evaluated to prevent dehydration. Presence of nodulation was scored by counting the nodule number (NN), and nodule dry weight (NDW-mg) was estimated following desiccation at 60 ˚C for 48 hours. After harvesting the nodules, roots were scanned (Epson Perfection V 700, Regent Instruments Inc., QC) and analyzed using WinRhizo© (Regent Instruments Inc., QC) to determine total root length (TRL-cm), root average diameter (RAD-mm), total root surface area (TRSA-cm^2), and total root volume (TRV-cm^3). Roots were then dried at 70 ˚C for 72 hours and root dry weight (RDW-g) was recorded. Specific nodule weight (SpNDW) was estimated by dividing NDW by NN, as a measure of nodule size, and root to shoot ratio (R:S) was calculated using RDW and SDW. N-use efficiency was calculated in terms of N_2 fixed/grams of nodule weight. IG 116024 (*L. nigricans*) did not flower before the experiment was terminated therefore phenotypic evaluations were conducted 62 days after seeding (DAS) for this accession.

3.2.4.2. At maturity

Days to maturity (DTM) were recorded for each unit when about 90% of the pods were brown. Whole plants were harvested and dried at 50℃ for 72 hours. Seeds were separated, counted (SN) and weighed to determine seed weight (SW-g), and the rest of the plant was weighed to obtain plant dry weight. Harvest index (HI) was estimated as (SW/whole plant dry weight) × 100. Seed sub-samples (approx. 30-50 units) were ground to 2 mm with a seed grinder (Cyclone Sample Mill, Seedburo Co., Chicago, IL) and seed percentage protein was determined on 100 mg samples using a LECO analyzer (LECO FP628, Leco Corporation, Saint Joseph, MI). Thousand seeds weight (KSW) was estimated as (SW/SN) × 1000.

3.3 RESULTS

3.3.1 Nodulation and N fixation at flowering.

All accessions evaluated with the inoculated treatment presented nodules. Cultivated accessions had significantly higher numbers of nodules (NN) that ranged from 23 to 451 nodules per plant (Table 3.2), with an average of 233.1 among all *L. culinaris* accessions. Wild accessions had lower NN values that ranged from 5 to 172 per plant, with an average 51.9.

Table 3.2. Number of nodules, nodule dry weight (g), specific nodule weight (mg) and N_2 fixed (mg/plant) calculated using the N difference method, for 36 *Lens* species accessions inoculated with the strain BASF 1435 Nodulator XL® (*Rhizobium leguminosarum* bv. *viciae*) (mean±SD).

Accession	Species	Number of nodules	Nodule dry weight (mg)	Specific nodule dry weight (mg)	N_2 fixed[a] (mg/plant)
CDC Maxim	*L. culinaris*	451 (±191)	85.0 (±37.1)	0.19 (±0.05)	62.3 (±30.8)
CDC Redberry	*L. culinaris*	436 (±139.6)	129.1 (±54.9)	0.29 (±0.04)	72.4 (±23.4)
CDC Robin	*L. culinaris*	192 (±86.4)	58.0 (±27.8)	0.30 (±0.08)	77.2 (±18.3)
CDC Milestone	*L. culinaris*	129 (±13.4)	33.4 (±12.4)	0.25 (±0.07)	76.3 (±30.6)
CDC Asterix	*L. culinaris*	189 (±57.5)	49.7 (±16.8)	0.28 (±0.11)	86.6 (±32.2)
CDC KR-1	*L. culinaris*	364 (±96.7)	77.5 (±31.8)	0.20 (±0.05)	93.1 (±31.8)
CDC Greenstar	*L. culinaris*	301 (±90.3)	104.4 (±49.6)	0.32 (±0.09)	91.5 (±38.9)
CDC QG-4	*L. culinaris*	216 (±143.8)	38.9 (±28)	0.13 (±0.04)	50.6 (±16.7)
Eston	*L. culinaris*	206 (±53)	28.8 (±12.8)	0.13 (±0.04)	35.6 (±6.7)
VIR-421	*L. culinaris*	168 (±47.5)	27.5 (±11.7)	0.16 (±0.03)	26.0 (±15.5)
Lupa	*L. culinaris*	23 (±12.0)	1.8 (±0.6)	0.07 (±0.01)	3.6 (±1.8)
ILL 7502	*L. culinaris*	99 (±59.8)	17.7 (±3.7)	0.25 (±0.12)	29.0 (±12.1)
ILL 1704	*L. culinaris*	153 (±64.1)	22.6 (±3.8)	0.17 (±0.05)	19.5 (±9.1)
ILL 8006	*L. culinaris*	268 (±89.4)	58.7 (±14.7)	0.23 (±0.04)	33.0 (±13.9)
Indianhead	*L. culinaris*	294 (±76.9)	60.8 (±11.3)	0.23 (±0.09)	82.0 (±30.4)
BGE 016880	*L. orientalis*	11 (±8.9)	16.8 (±2.4)	0.25 (±0.05)	5.5 (±7.6)
IG 72529	*L. orientalis*	19 (±2.9)	3.5 (±0.8)	0.20 (±0.09)	1.5 (±0.43)
IG 72611	*L. orientalis*	74 (±17.8)	17.4 (±10.6)	0.18 (±0.08)	7.9 (±3.1)
IG 72622	*L. orientalis*	36 (±12.2)	11.6 (±4.8)	0.38 (±0.11)	4.5 (±2.8)
IG 72643	*L. orientalis*	39 (±15.2)	12.4 (±7.4)	0.33 (±0.11)	18.6 (±7.1)
IG 72672	*L. orientalis*	18 (±8.2)	5.7 (±3.44)	0.43 (±0.13)	0.6 (±0.29)
PI 572376	*L. orientalis*	5 (±1.5)	4.1 (±1.6)	0.94 (±0.3)	3.5 (±2.7)
IG 72613	*L. tomentosus*	46 (±19.1)	10.5 (±4.0)	0.23 (±0.02)	4.7 (±2.4)
IG 72614	*L. tomentosus*	19 (±6.8)	4.3 (±1.63)	0.27 (±0.12)	0.6 (±0.2)
IG 72805	*L. tomentosus*	18 (±7.6)	6.7 (±1.9)	0.43 (±0.13)	7.1 (±3.2)

Table 3.2. Continued.

Accession	Species	Number of nodules	Nodule dry weight (mg)	Specific nodule dry weight (mg)	N_2 fixed[a] (mg/plant)
PI 572390	*L. tomentosus*	39 (±4.6)	8.9 (±1.2)	0.21 (±0.01)	11.0 (±6.1)
IG 72543	*L. odemensis*	45 (±14.2)	9.3 (±3.3)	0.20 (±0.02)	6.3 (±2.7)
IG 72623	*L. odemensis*	143 (±76.2)	20.5 (±11.1)	0.14 (±0.03)	12.4 (±6.2)
IG 72760	*L. odemensis*	172 (±12.7)	31.0 (±8.1)	0.18 (±0.04)	35.2 (±15.6)
IG 110810	*L. lamottei*	17 (±5.7)	7.2 (±1.7)	0.46 (±0.09)	6.1 (±1.9)
IG 110813	*L. lamottei*	18 (±3.8)	5.1 (±3.2)	0.3 (±0.11)	4.7 (±2.3)
IG 72537	*L. lamottei*	12 (±3.2)	4.9 (±2.6)	0.46 (±0.19)	1.5 (±0.4)
IG 72815	*L. ervoides*	67 (±27.1)	7.2 (±3.6)	0.11 (±0.02)	3.4 (±1.4)
L01-827A	*L. ervoides*	13 (±6.4)	8.1 (±3.7)	0.7 (±0.21)	3.3 (±1.3)
LR59-81	interspecific[b]	127 (±81.2)	19.4 (±9.4)	0.1 (±0.01)	10.6 (±6.3)
IG 116024	*L. nigricans*	78 (±15.8)	25.6 (±6.1)	0.3 (±0.12)	9.9 (±5.8)
LSD		114.2***	29.72***	0.28***	25.56***

ANOVA values are ns: not significant, *, **, ***: significant at 0.05, 0.01, 0.001 respectively. [a]N_2 fixed estimated by difference method for R plot (Total N accumulation R plot - Total N accumulation C plot). [b]*L. culinaris* × *L. ervoides.* White: cultivated and grey: wild accessions.

Lupa and ILL 7502 were the only cultivated accessions with low NN, similar to those found in the wilds. From the wilds, only IG 72623 (*L. odemensis*) and IG 72760 (*L. odemensis*) had NN that were not different from some of the cultivated accessions.

Nodule dry weight (NDW) was significantly different among cultivated and wild accessions (Table 3.2). CDC Redberry and CDC Greenstar were among the genotypes with the highest NDW values. Cultivated accessions Lupa, Eston, VIR-421, ILL 1704 and ILL 7502 had NDW means that were not different from those observed for all wilds. NDW was not different among the wild accessions, including those with higher NN. Specific nodule dry weight (SpNDW) was higher only in a few wild accessions: PI 572376 (*L. orientalis*), L01-827A (*L. ervoides*), IG 72537 and IG 110810 (*L. lamottei*). Efficiency of nodulation (N fixed per g of nodule weight) was not significantly different among accessions. Different nodule shapes were observed and will be covered in Chapter 5.

N fixed observed per plant (mg) had a great range of values (Table 3.2) especially for the cultivars: from 1.8-129.13 mg/plant and 54.4 mg/plant on average. Among the wild accessions, values 3wranged from 0.6-35.2 mg/plant and 8.55 mg/plant on average. Cultivars Eston, VIR-

421, ILL 7502, ILL 1704, ILL 8006 and Lupa fixed similar amounts of N as those fixed by the wild accessions.

3.3.2. Effects of inoculation on seed traits and harvest index.

A significant effect of *Rhizobium* (R) compared to added Nitrogen (+N) treatment, and differences among accessions were observed for all seed parameters evaluated (Table 3.3), as well as some specific accession × treatment interactions. Seed number (SN) was lower with R compared to +N for the accessions CDC Maxim, BGE 016880 (*L. orientalis*) and L01-827A (*L. ervoides*). Lower seed weight (SW) values were observed under R for the cultivated accessions CDC Maxim and CDC KR-1. No differences were found for SW of CDC Greenstar or Indianhead under +N vs. R. Lupa had the lowest SW observed among the cultivated accessions under R compared to +N. PI 572376 (*L. orientalis*) had more than twice the SN under +N compared to R, and was the only wild accession with a lower SW under R. All other wilds had SW values that were similar under R and +N.

For thousand seed weight (KSW) (Table 3.3), CDC Greenstar had a higher value under R treatment compared to +N. No differences based on sources of N were observed among the other accessions for this trait. *Rhizobium* inoculation had no impact on harvest index (HI) among the cultivated accessions, but IG 72643 (*L. orientalis*), PI 572390 (*L. tomentosus*), IG 72623 (*L. odemensis*) and IG 110810 (*L. lamottei*), significantly increased their HI value under R treatment. PI 572390 (*L. tomentosus*) and IG 110810 (*L. lamottei*) had HI increases of 16 and 14% respectively under the R treatment compared to +N.

Seed percentage protein was the seed trait with the most variation observed under R treatment when compared to plants with +N treatment (Table 3.3). CDC Maxim, CDC Greenstar and Lupa had lower values when inoculated with R.

Table 3.3. Seed number, seed weight (g), seed percentage protein, thousand seed weight (g), and harvest index of 14 *Lens* species accessions with the strain BASF 1435 Nodulator XL® (*Rhizobium leguminosarum* bv. *viciae*) and added Nitrogen treatments (mean ± SD).

Accession	**Seed number**		**Seed weight (g)**		**Seed percentage protein**	
	+Nitrogen	***Rhizobium***	**+Nitrogen**	***Rhizobium***	**+Nitrogen**	***Rhizobium***
CDC Maxim	217.7 (±25.9)	124.3 (±34.1)	8.5 (±0.8)	5.3 (±1.1)	19.1 (±0.8)	17.0 (±0.7)
CDC KR-1	188.7 (±34.2)	136.5 (±37.1)	9.7 (±1.2)	6.8 (±1.5)	18.9 (±0.8)	18.5 (±0.8)
CDC Greenstar	139.7 (±8.8)	125.0 (±18.9)	9.8 (±0.7)	9.4 (±1.6)	19.4 (±0.7)	18.1 (±0.8)
Lupa	103.5 (±22.3)	43.0 (±9.5)	3.5 (±1.1)	**1.6 (±0.6)**	19.6 (±0.7)	17.9 (±0.6)
Indianhead	382.5 (±72.9)	371.2 (±60.2)	9.1 (±1.9)	9.8 (±1.4)	22.6 (±0.9)	25.3 (±1.0)
BGE 016880	208.5 (±47.5)	105.3 (±23.4)	2.9 (±0.8)	1.8 (±0.4)	20.3 (±0.8)	23.8 (±0.9)
IG 72643	279.7 (±78.1)	207.7 (±42.3)	3.9 (±1.4)	2.9 (±0.7)	21.8 (±0.8)	**26.4 (±1.0)**
PI 572376	**372.2** (±43.4)	169.6 (±36.2)	2.7 (±1.3)	1.3 (±0.6)	22.5 (±0.9)	21.5 (±0.9)
PI 572390	226.0 (±51.2)	236.3 (±48.5)	2.9 (±0.6)	3.2 (±0.8)	20.8 (±0.8)	**26.2 (±1.0)**
IG 72623	357.0 (±71.9)	329.0 (±70.9)	5.5 (±1.8)	5.0 (±1.6)	24.9 (±1.0)	24.5 (±1.0)
IG 72760	178.5 (±80.2)	141.3 (±56.4)	2.2 (±0.9)	1.7 (±0.7)	19.4 (±0.8)	19.2 (±0.8)
IG 110810	174.8 (±65.0)	123.2 (±25.8)	2.6 (±0.8)	2.2 (±0.9)	26.2 (±1.1)	25.8 (±1.0)
L01-827A	303.7 (±57.3)	186.8 (±17.9)	2.1 (±0.4)	1.2 (±0.1)	22.1 (±0.8)	24.1 (±0.9)
LR59-81	176.5 (±73.7)	244.7 (±50.2)	2.6 (±1.2)	2.3 (±0.8)	25.7 (±1.1)	26.5 (±1.1)
Mean	236.4	174.6	4.9	3.8	25.7	26.5
LSD	81.3^{***}		1.3^{***}		0.7^{***}	

Table 3.3. Continued.

Accession	Thousand seeds weight (g)		Harvest index (%)	
	+Nitrogen	***Rhizobium***	**+Nitrogen**	***Rhizobium***
CDC Maxim	39.1 (±1.9)	42.3 (±2.9)	50 (±2.6)	47 (±4.9)
CDC KR-1	52.0 (±8.1)	50.6 (±6.3)	53 (±4.6)	56 (±3.1)
CDC Greenstar	70.0 (±4.4)	**75.8** (±6.6)	55 (±4.8)	53 (±6.8)
Lupa	33.1 (±3.7)	35.6 (±3.1)	43 (±10.5)	40 (±7.8)
Indianhead	23.8 (±1.1)	26.6 (±2.7)	52 (±4.9)	49 (±2.6)
BGE 016880	13.8 (±1.6)	16.6 (±1.0)	37 (±7.1)	36 (±1.9)
IG 72643	14.4 (±3.2)	14.0 (±2.2)	35 (±7.9)	45 (±1.6)
PI 572376	7.4 (±0.6)	7.9 (±0.4)	34 (±5.2)	37 (±6.8)
PI 572390	13.0 (±1.6)	13.8 (±1.2)	37 (±8.2)	**53** (±7.8)
IG 72623	14.8 (±1.4)	15.6 (±1.1)	40 (±7.7)	48 (±2.4)
IG 72760	12.1 (±0.6)	12.0 (±0.3)	43 (±10.9)	46 (±8.9)
IG 110810	15.1 (±1.0)	17.2 (±3.9)	35 (±3.7)	**49** (±9.3)
L01-827A	6.9 (±0.2)	6.5 (±0.3)	43 (±1.7)	44 (±2.0)
LR59-81	14.4 (±2.0)	15.5 (±0.7)	26 (±5.4)	27 (±4.2)
Mean	23.6	25.0	42	45
LSD	4.4^{***}		7.6^{***}	

ANOVA values are ns: not significant, *, **, ***: significant at 0.05, 0.01, 0.001 respectively. **Bolded** numbers represent the significant interactions for accession × plot (+Nitrogen/*Rhizobium*). White: cultivated and grey: wild accessions.

In contrast, Indianhead, BGE 016880 (*L. orientalis*), PI 572390 (*L. tomentosus*), IG 72643 (*L. orientalis*), IG 72623 (*L. odemensis*), L01-827A (*L. ervoides*) and LR59-81 (interspecific) increased this value with R compared to +N. IG 72643 (*L. orientalis*) and PI 572390 (*L. tomentosus*) had the biggest percentage protein in seed increases when inoculated with R.

3.3.3. Effects of inoculation on plant growth and N accumulation at flowering.

Rhizobium inoculation (R) delayed flowering (DTF) by 2 days on average compared to +N treatment for both cultivated and wild accessions, with a few exceptions (Figure 3.1 A). Cultivated accessions CDC Milestone and ILL 1704 did not flower later when inoculated (R) compared to +N treatment. Wild accessions BGE 016880 (*L. orientalis*), IG 72760 (*L. odemensis*), PI 572376 (*L. orientalis*) and PI 572390 (*L. tomentosus*) also flowered at the same time under both R and +N treatments. IG 72622 (*L. orientalis*) was the only accession that flowered later with the +N treatment compared with the R treatment.

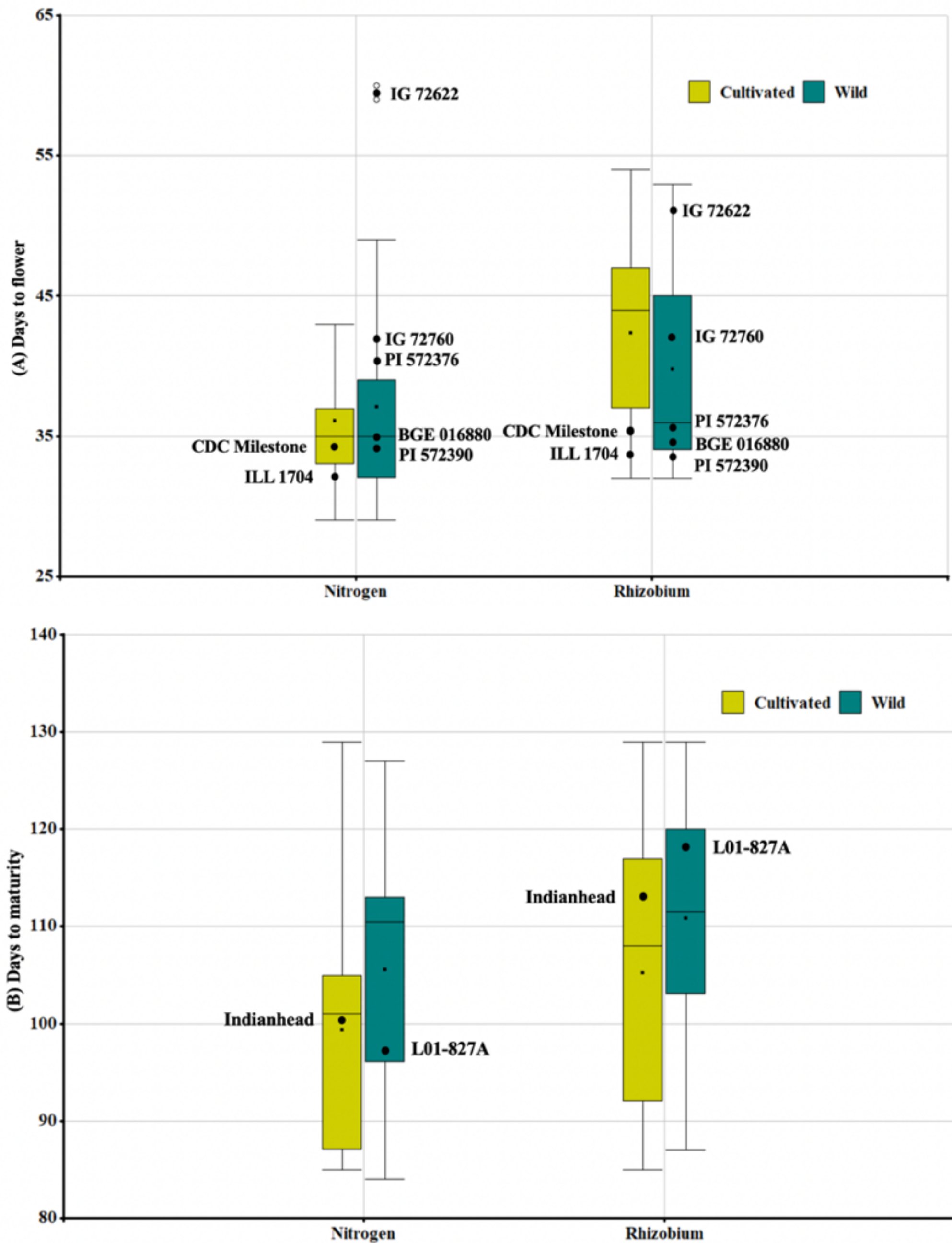

Figure 3.1. A: days to flower (LSD 4.8, P=0.01) of 36 *Lens* species accessions and B: days to maturity (LSD 9.6, P=0.01) of 14 *Lens* species accessions, with added Nitrogen or BASF 1435 Nodulator XL® (*Rhizobium leguminosarum* bv. *viciae*) treatments. Means of cultivated accessions (light green boxplots) are displayed separately from the wild ones (dark green boxplots).

For the sub-group of 14 accessions studied until maturity (Table 3.1), the average days to maturity (DTM) were not different for R and +N treatments (Figure 3.1 B). Among the genotypes, only Indianhead and L01-827A (*L. ervoides*) experienced delays of 11.7 and 18.2 days, respectively when inoculated with *Rhizobium* (See Table B, Appendix B for specific DTF and DTM values for all genotypes, Table C, Appendix C for ANOVA and LSD results).

For traits directly related to the amount of N fixed, means observed in the C treatment are also presented. Mean shoot dry weight (SDW) was the highest under the +N treatment for both cultivated and wilds (Figure 3.2 A). Wild accessions had lower weights under all treatments. The R treatment mean was significantly lower and fewer differences were observed among the wild accessions compared to the differences found among cultivated ones. Shoot weights in the control had lower values compared to +N and R treatments for the cultivated accessions, but wilds had the same weights for R and C treatments.

No differences were observed for the accessions CDC Maxim, CDC Redberry, CDC Robin, CDC KR-1and CDC Greenstar under +N and R treatments for SDW (Figure 3.2 A).

Root dry weight (RDW) values under the +N treatment (Figure 3.2 B), were also significantly higher compared to those observed in the R treatment. CDC Maxim and BGE 016880 (*L. orientalis*) had values of RDW that were not different under +N and R treatments. Most of the cultivated accessions exhibited different values between R and C treatments for this trait, except CDC Redberry, CDC Asterix, CDC QG-4 and Eston. All wild accessions had similar values of RDW under R and C treatments.

Treatments were not different in their overall root to shoot ratios (R:S) (Figure 3.2 C). Among accessions, IG 110810 (*L. lamottei*) had higher ratios with R compared to +N. BGE 016880 (*L. orientalis*) had a higher R:S value with R compared to both +N and C. CDC Redberry, CDC Robin and CDC KR-1, had a higher R:S value under C compared to R. Similarly, higher values under the C treatment were observed for a group of the wild accessions, including IG 72643 (*L. orientalis*), PI 572390 (*L. tomentosus*), L01-827A (*L. ervoides*) when compared to R treatment. (See Table D.1, Appendix D for all SDW, RDW and R:S means).

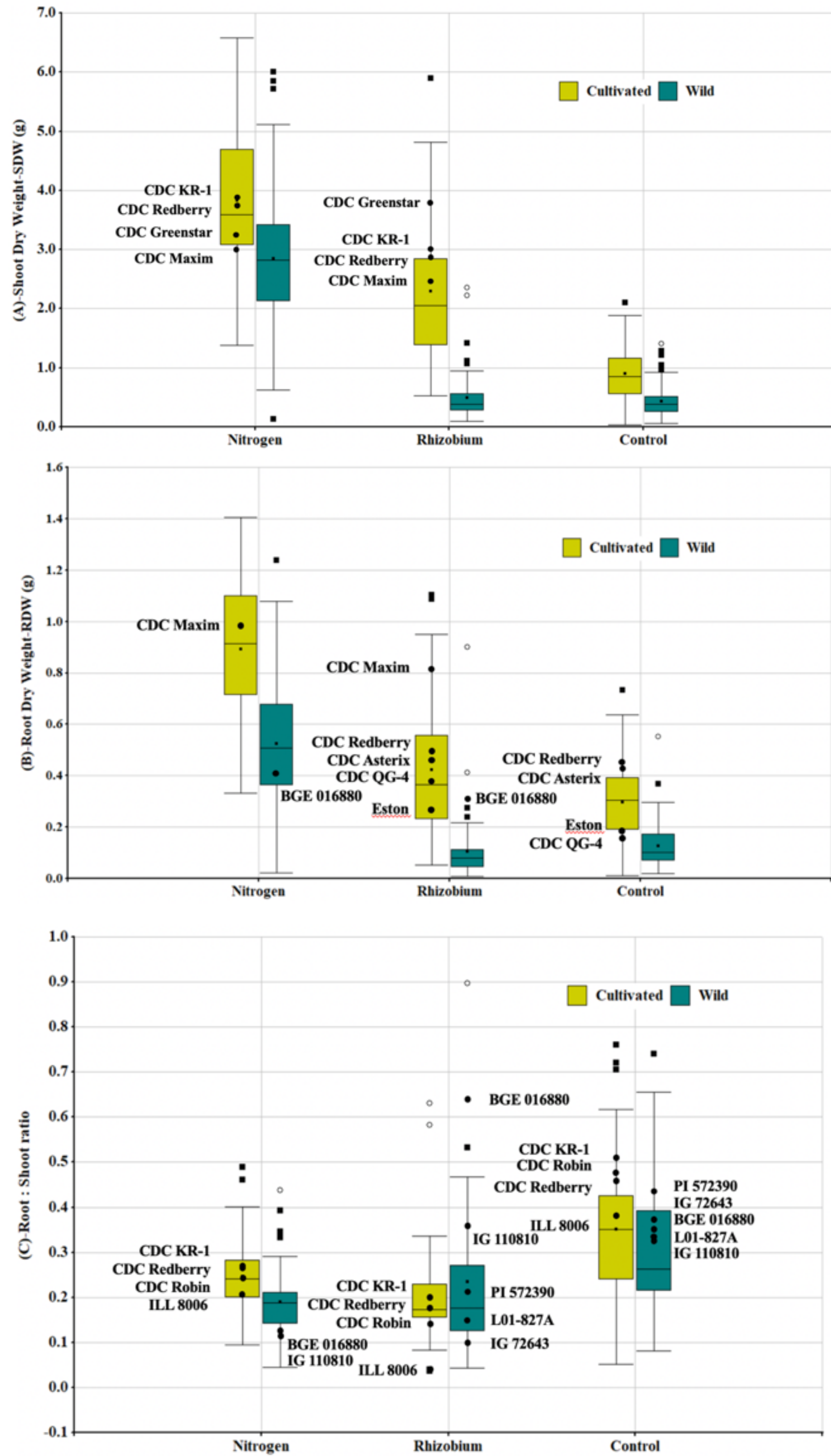

Figure 3.2. A: shoot dry weight (LSD 0.96, P=0.01), B: root dry weight (LSD 0.21, P=0.01) and C: root to shoot ratio (LSD 0.2, P=0.01), of 36 cultivated and wild *Lens* species accessions with added Nitrogen, BASF 1435 Nodulator XL® (*Rhizobium leguminosarum* bv. *viciae*) and Control treatments. Means of cultivated accessions (light green boxplots) are displayed separately from the wild ones (dark green boxplots).

N concentration in shoots was different under all three treatment (Figure 3.3). Overall R had the highest N concentration in shoots at flowering for both cultivated and wilds. Accessions Lupa, ILL 1704, ILL 8006, BGE 016880 (*L. orientalis*), IG 72614 (*L. tomentosus*) and IG 72543 (*L. odemensis*) were not different between their R and +N treatments, however (See Table D.2, Appendix D for specific N concentration values for all genotypes). N concentration in shoots was in general significantly lower in the C treatment than both +N and R treatments, although the accessions Lupa, IG 72611 (*L. orientalis*), IG 72537 (*L. lamottei*) and L01-827A (*L. ervoides*) values in the C treatment did not differ from those observed in the R treatment.

Measurements of chlorophyll content taken with the SPAD meter had a low correlation with the measurements obtained with the Leco analyzer (Figure E, Appendix E), therefore were not taken into consideration for estimating N accumulation.

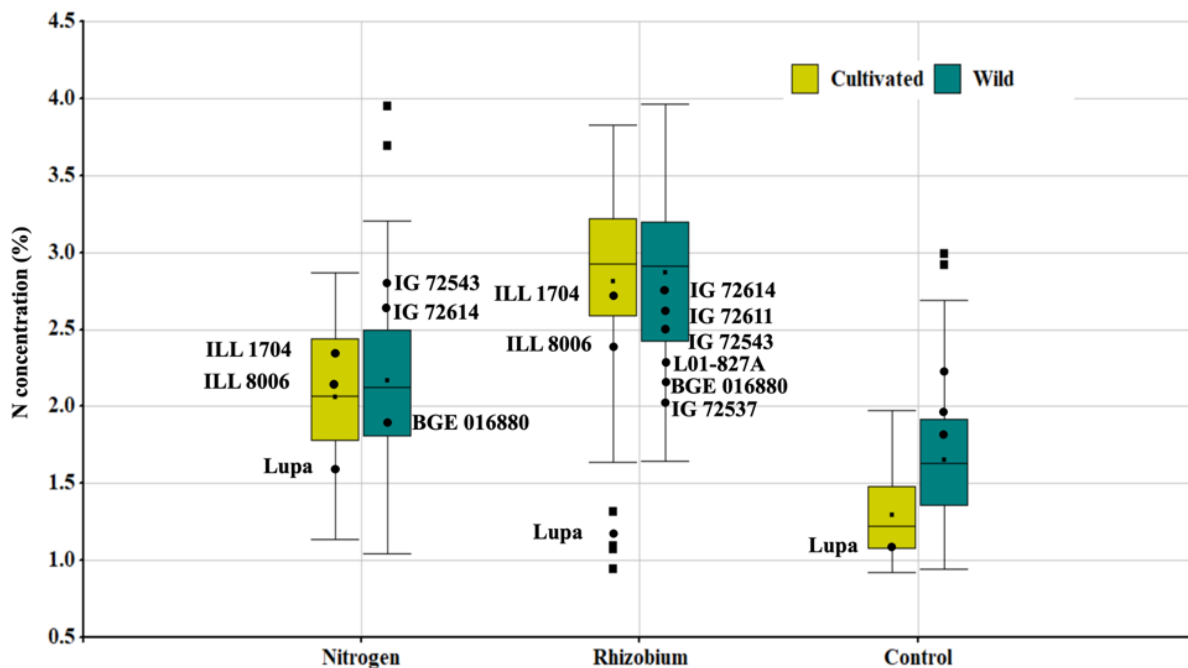

Figure 3.3. N concentration in shoots (%) of 36 cultivated and wild *Lens* species accession with added Nitrogen, BASF 1435 Nodulator XL® (*Rhizobium leguminosarum* bv. *viciae*) and Control treatments (LSD= 0.52, P=0.01). Means of cultivated accessions (light green boxplots) are displayed separately from the wild ones (dark green boxplots).

There was a tendency among the cultivated accessions to have a bigger proportion of N fixed, related to the mineral N obtained from media as measured in the C treatment compared to most wilds (Figure 3.4). Lupa accumulated the same proportion of N fixed as the wilds, and from the

wilds, IG 72760 (*L. odemensis*) had similar proportions of N fixed to the average of the cultivated accessions. CDC Robin and CDC Milestone were among the cultivated accessions with the highest proportion of N obtained from fixation. IG 72529 (*L. orientalis*), IG 72672 (*L. orientalis*) and IG72614 (*L. tomentosus*) were among the wilds with the lowest proportion of N obtained from fixation.

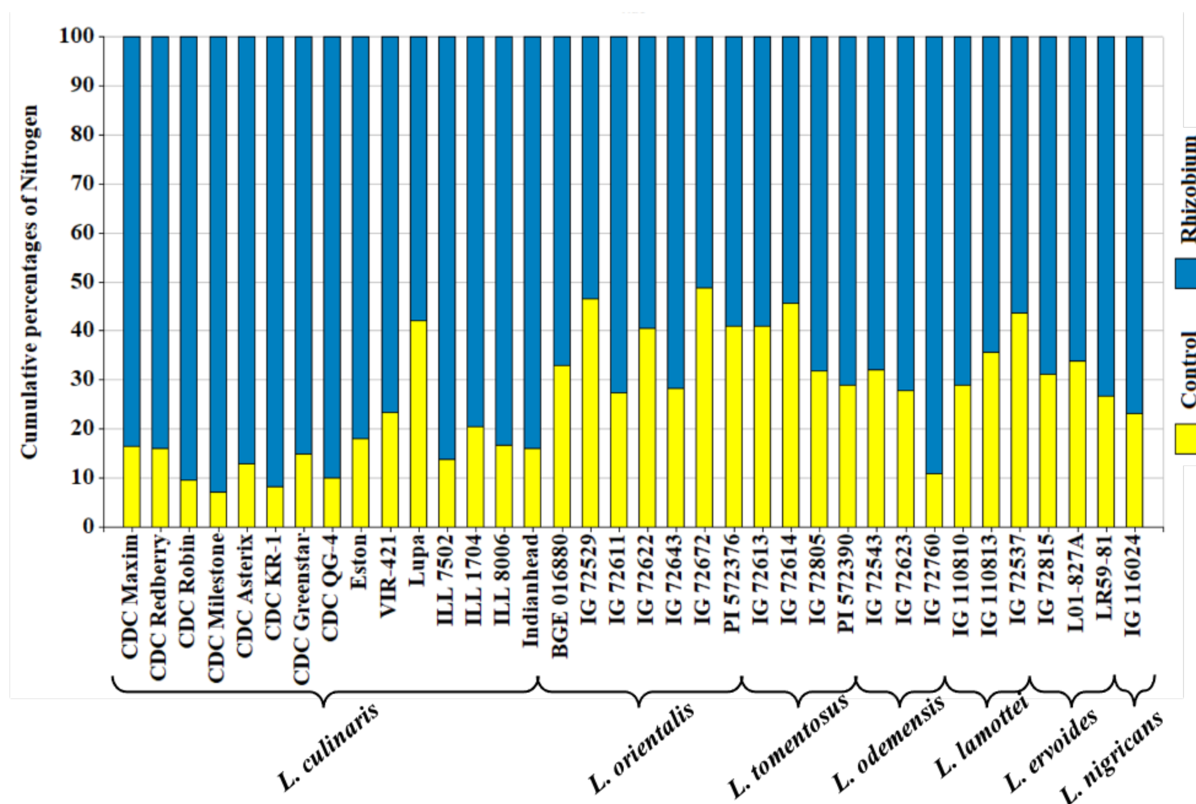

Figure 3.4. Cumulative percentage of Nitrogen. Total N accumulation= N_2 fixed (N yield *Rhizobium* plot- N yield Control plot) + N yield Control plot. N yield Control plot was the mineral N on media.

3.3.4. Effects of inoculation on root distribution

All the root parameters evaluated were overall higher under +N compared to the roots observed under the R treatment, and similar between R and C treatments (Figure 3.5). For the total root length (TRL), CDC Maxim and CDC Robin were not different between +N and R. Cultivated accessions CDC Maxim, CDC Robin, CDC Milestone, CDC KR-1, CDC Greenstar and Indianhead were the only ones to show differences under the R and C treatments. L01-827A (*L. ervoides*) had similar TRL under +N and C, and IG 72815 (*L. ervoides*) had similar TRL in all treatments.

Total root volume (TRV) was different between roots observed with +N and R, except for CDC Maxim, IG 72543 (*L. odemensis*) and IG 110810 (*L. lamottei*). Values of TRV were similar under the R and C treatment except for the group of cultivated accessions: CDC Maxim, CDC Robin, CDC Milestone, CDC KR-1 and CDC Greenstar. TRV of L01-827A *(L. ervoides*) and IG 72815 (*L. ervoides*) were not different under any treatment (Figure 3.5).

Total root surface area (TRSA) observed between +N and R treatments was only similar for CDC Maxim and IG 110810 (*L. lamottei*) and the rest presented significantly lower values of TRSA under R compared to +N (Figure 3.5). Most accessions had similar values under R and C. Only the group of cultivated accessions CDC Maxim, CDC Robin, CDC Milestone, CDC KR-1, CDC Greenstar and Indianhead, had values of TRSA different between R and C treatments. IG 72543 (*L. odemensis*) was not different under +N and C treatments. IG 72815 (*L. ervoides*) and L01-827A (*L. ervoides*) did not differ on any of the treatments for this parameter.

Root average diameter (RAD) was also similar between +N and R only for a number of accessions: CDC Maxim, CDC Asterix, CDC KR-1, IG 72543 (*L. odemensis*) and IG 72760 (*L. odemensis*). The rest of accessions had higher RAD under +N compared to R. RAD observed under R and C treatments was only similar for the accessions CDC Robin, CDC Milestone and CDC Greenstar, and the rest of accessions exhibited similar RAD under both treatments. CDC Asterix and IG 72543 (*L. odemensis*) were not different under +N and C treatments on their RAD, and IG 110810 (*L. lamottei*), IG 72815 (*L. ervoides*) and L01-827A (*L. ervoides*) were not different under any treatment for RAD.

In general, most of the accessions responded with higher values for all root parameters with +N treatment. Differences observed between R and C treatments were among the cultivated ones, but none of the wild accessions had differences for any of the parameters under these two treatments (See Table F, Appendix F for TRL, TRV, TRSA and RAD means).

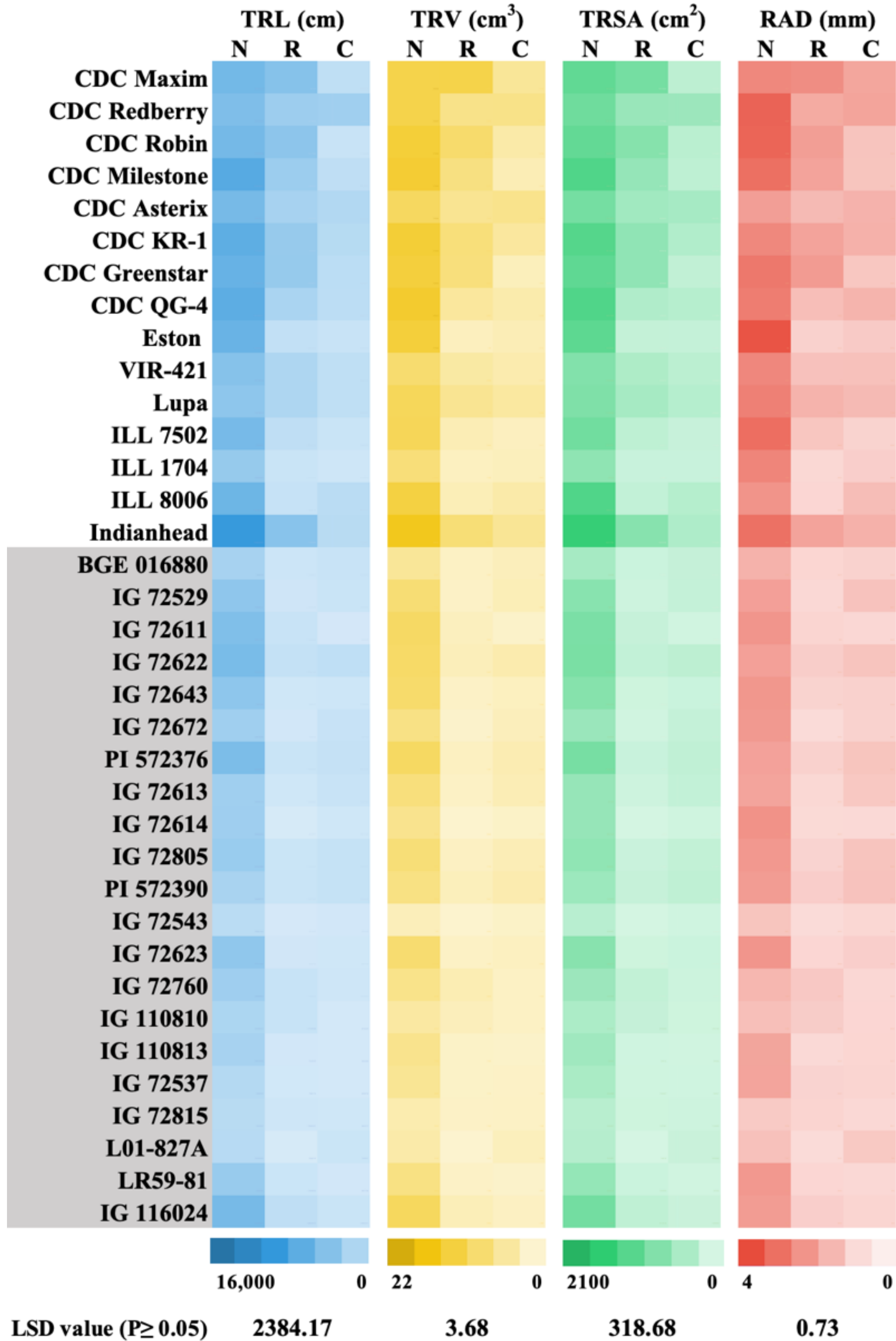

Figure 3.5. Heatmap for total root length (TRL), total root volume (TRV), total root surface area (TRSA) and root average diameter (RAD) of 36 *Lens* species accessions with added Nitrogen (N), BASF 1435 Nodulator XL® (*Rhizobium leguminosarum* bv. *viciae*) (R) and Control (C) treatments. White: cultivated and grey: wild accessions.

3.3.5. Correlations among evaluated parameters.

Values presented in Figure 3.6 correspond to correlation coefficients of the 14 accessions evaluated up to maturity to understand the relationships among parameters evaluated at flowering and maturity. Correlations among the 36 accessions evaluated at flowering showed similar results among traits evaluated at flowering (Figure G, Appendix G).

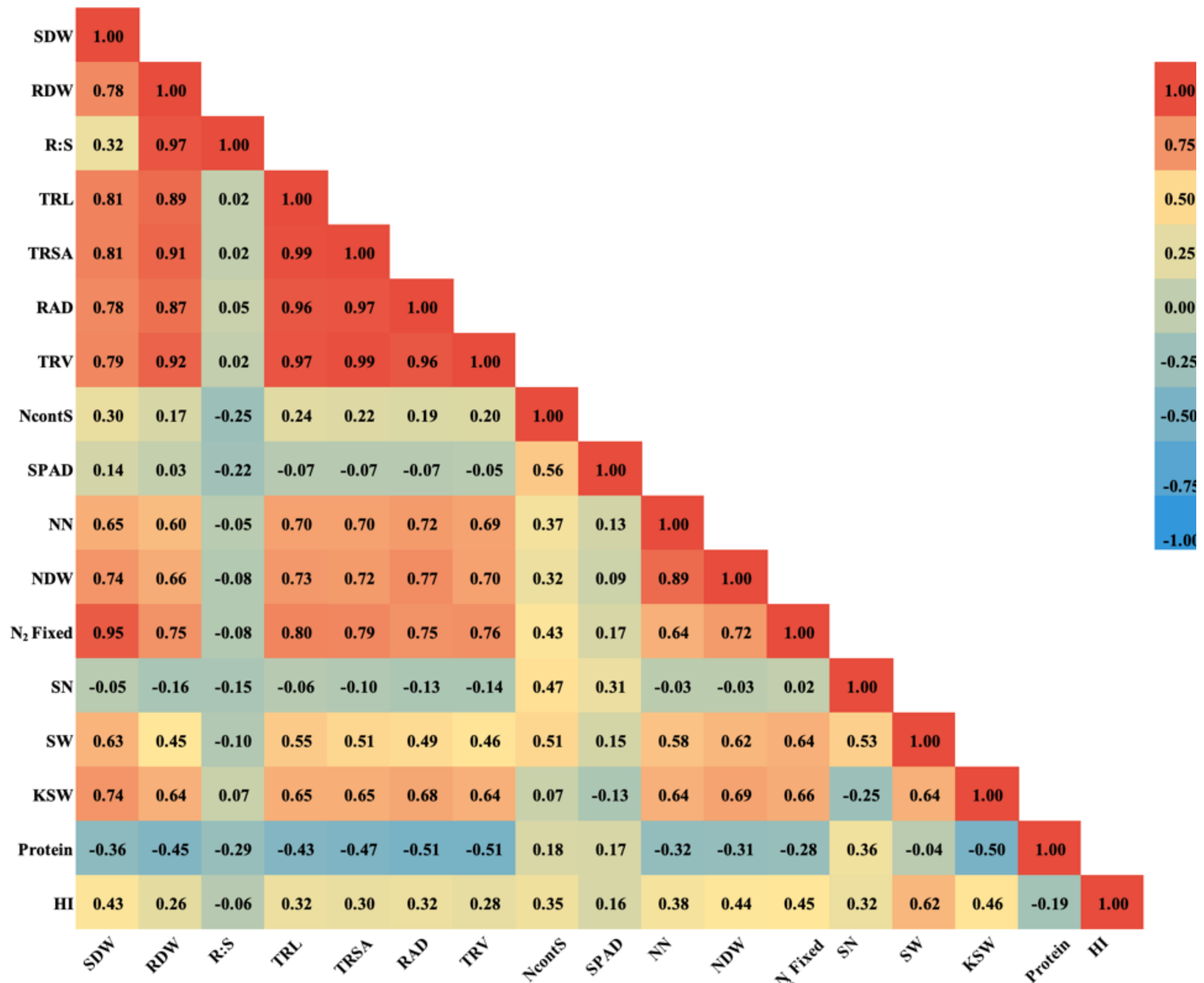

Figure 3.6. Pearson correlation coefficients among parameters evaluated on 14 *Lens* species accessions at flowering and maturity, when inoculated with BASF 1435 Nodulator XL® (*Rhizobium leguminosarum* bv. *viciae*). SDW: shoot dry weight, RDW: root dry weight, R:S: root to shoot ratio, TRL: total root length, TRSA: total root surface area, RAD: root average diameter, TRV: total root volume, NcontS: N concentration in shoots, SPAD: leaf chlorophyll concentration, NN: number of nodules, NDW: nodule dry weight, N_2 fixed, SN: seed number, SW: seed weight, KSW: thousand seed weight, Protein: seed percentage protein and HI: harvest index.

Shoot dry weight (SDW) was highly correlated with RDW, all root parameters measured with WinRhizo© (TRL, TRSA, RAD and TRV), NDW and N fixed (Figure 3.6). RDW had the strongest correlation with the root parameters, NDW and N fixed. There was no correlation between N concentration in shoots and SPAD measurements and neither was correlated with any other parameter.

Root parameters were highly correlated with NN, NDW, N fixed and KSW. Nodulation parameters NN and NDW and N fixed, were also correlated to KSW. N fixed had the strongest correlation with SDW and NDW. SW was correlated with SDW, NDW and N fixed. R:S was only correlated to RDW. N concentration in shoots, SN and seed percentage protein were not correlated with any other measured parameter.

3.4. DISCUSSION

Exploring a greater diversity of *Lens* spp. accessions was necessary to identify the best resources within the genus *Lens* to improve the N fixing ability of lentil. The selected group of cultivated accessions are representative of the genetic material generated by the Crop Development Centre at the University of Saskatchewan and also some accessions representing other areas of the world. It also included wild accessions from 6 species from the center of origin of lentils that are already part of the breeding program or are being characterized. The purpose of the study was to identify any potential species or specific accessions with characteristics related to higher symbiotic ability.

Both cultivated and wilds displayed broad differences and trends did not correspond to any specific *Lens* species, but this group exhibited some of the differential mechanisms that exist between wild and cultivated plants in their N fixing process and how this impacts seed production. The phenotypic nodulation system, N fixed and N translocation, root adaptation mechanisms in inoculated plants, protein content and seed yield were the major dissimilarities found between wilds and cultivars.

All vegetative measurements as well as N fixed, were taken at reproductive stage 1 (R1) just as flowering started. From R1 to R3, more flowers and vegetative structures continue to develop. CDC Greenstar and Indianhead were among the highest N fixers at flowering with values of fixation above the average for all cultivated accessions. Furthermore, they also had similar seed

weight, thousand seeds weight and harvest index when inoculated. Conversely, CDC KR-1 that was within the highest N fixers at flowering, yielded similar to CDC Maxim and Lupa that were intermediate and poor N fixers respectively. In contrast, wild accessions kept similar seed weights regardless of the amount of N fixed at flowering, except for PI 572376 (*L. orientalis*). The consistent yields did not have any negative effects on seed size, protein content nor harvest index, rather some higher protein content and harvest index were observed. IG 72623 (*L. odemensis*) in particular, fixed 12.4 mg of N at flowering and CDC Maxim did 62.3 mg, but both yielded the same. Results of N accumulation in this study suggest that wild *Lens* spp. have a higher ability to efficiently use N compared to cultivars. Studies conducted with 14 cultivated crop species show that only four cereals have higher ability to use N compared to their wild relatives, but most of the cultivated crops have not outdone their wild relatives (Rotundo and Cipriotti 2016).

For the accessions evaluated up to maturity (R8), the Pearson correlation coefficient between N fixed and seed weight (SW) was 0.64 (Figure 3.6), a moderate relationship between the two variables. In fact, this was the highest correlation observed between SW and any other parameter measured at flowering, suggesting that selection of genotypes for better N fixing ability cannot be based solely on their phenotypic expression at flowering. Ultimately, what determines a genotype's superior ability to assimilate N is seed production (Unkovich *et al*., 2008). In addition to this, the higher HI (Table 3.3) identified among wild accessions (from 4 species) suggests they have a higher efficiency in translocating N to seed. Wild accessions also had overall higher protein concentration in seeds, and at the same time, their protein values were increased when inoculated with *Rhizobium*. Transfer from wild related species of legumes of a higher ability to obtain and translocate N is already known. Successful increases in protein content of about 15 % in seeds have been obtained in pigeon pea (*Cajanus cajan*) through introgression from wild relatives (Saxena *et al*., 2002). The mechanisms through which they are able to translocate more N in symbiosis, compared to when they received added N, has to be further studied in lentils. One possibility could be the differential nodulation observed on wild species provides the plant with a N source during flowering and early maturity, in contrast to the nodule phenotypes observed in cultivars that reduce their activity earlier during early maturing.

N fixed values were estimated based on a comparison of each genotype relative to its own reference plant. It is a reliable technique because was conducted under low N controlled conditions. However, genotypes variated in their mechanisms to acquire N, as a consequence, N fixing and reference plants do not remove identical amounts of N from the media. N fixing plants can obtain their N from added N absorbed through roots, fixed N from their bacterial symbiont, and, under very low N availability, organic sources may be accessed from the soil (Lipson and Näsholm 2001). In this experiment the control treatment (C) was expected to contain small amounts of this organic N. As shown in Figure 3.4, the percentage of N acquired in the Control treatment, in relation to the percentage acquired from the symbiont, had a tendency to be greater in most of the wilds, with the exception of IG 72760 (*L. odemensis*), as a consequence, its N fixed estimate is greater than all the other wild accessions.

It was important to identify the root adaptation mechanisms specific genotypes utilized under N limiting conditions and how this affects N acquisition. Plants responded to changing N resources by altering allocation biomass patterns. More root biomass allocation when lower water or nutrients are available is one of mechanisms plants employ to tolerate these types of stresses (Ågren and Ingestad 1987). In lentil, genotype-specific mechanisms of root allocation have been observed under drought conditions (Gorim and Vandenberg 2017). The significantly higher R:S ratio of some accessions in the controls (Figure 3.2 C, Table D.1, Appendix D) reflects the differential ability of genotypes to use low organic N sources. Those with higher ratios manage to allocate more root biomass to extract more of the N available in the growing media, as was the case of BGE 016880 (*L. orientalis*), IG 72643 (*L. orientalis*) and L01-827A *(L. ervoides*). Some of the cultivars including CDC Redberry and CDC KR-1 were also among those with higher R:S ratios. In contrast, CDC Maxim, kept the same R:S across treatments. Most cultivars, however, remove similar and low amounts of N regardless of how they allocated their biomass, which shows their inability to adapt to N deficient stress conditions.

The specific root parameters obtained with WinRhizo© (Figure 3.6, Table F, Appendix F), showed in more detail some of the architectural modifications in the root systems employed by a few accessions in response to the different treatments. Cultivated accessions had smaller values for all parameters with *Rhizobium* inoculation and as expected, under the Control treatment. In contrast, the wilds did not show differences between the *Rhizobium* inoculation and Control

treatments. While cultivars are larger plants in general, *Rhizobium* inoculation leads to even larger differences. Efficient N fixation makes allocation of root biomass independent of soil N supply (Markham and Zekveld 2007). The smaller root systems observed with *Rhizobium* inoculation, mainly in the wilds, compared to those observed with +N, suggests a more efficient N fixation. The higher allocation of root biomass in the cultivars, when inoculated with *Rhizobium* compared to +N, could also be a determining factor in the lower values of seed yield observed in cultivars but not in the wilds. Overall, the root parameters changed in proportion to RDW with no particular alteration of any specific root dimension under specific treatments. Of note, however, these differences in RDW among genotypes under the different treatments, are not related to a particular species and only distinctive to *L. culinaris*, suggesting that tolerance to low N has been reduced over the course of selection.

For all phenotypic nodulation parameters, differential phenotypes were observed between and within species. *L. culinaris* had the highest overall net values for NN (Table 3.2) and total N accumulation (Table D.2, Appendix D), with CDC varieties having the greatest values among the cultivars. These accessions have all been selected in the same region where this *Rhizobium* strain was inoculated for at least 2 decades. Better symbiotic results have been observed in lentils and other crops when locally adapted strains are used (Kurdali *et al*., 1997, Ferreira and Hungria 2017). Other *L. culinaris* selected elsewhere, including VIR-421 and the ILL lines performed poorly with this strain. Lupa, a Spanish variety, was particularly inefficient and was the only cultivated accession with undesirable nodulation characteristics in general.

IG 72623 (*L. odemensis*) and IG 72760 (*L. odemensis*) had nodulation characteristics similar to those observed on the cultivated lines in both numbers and phenotypic characteristics. However, the other wilds exhibited a range of phenotypic nodule diversity not observed in the cultivars, which was particularly reflected in higher specific nodule dry weight (SpNDW) values of some of them (Table 3.2). Those with higher SpNDW had bigger, indeterminate nodules. The wilds with lower SpNDW had both indeterminate and determinate nodules, which made their SpNDW values not different from those observed among the cultivated accessions. Determinate vs. indeterminate nodules have some fundamental structural differences that impact the bacteroid population. Determinate nodules consist of a homogeneous bacteroid population that synchronously senesces (Rolfe and Gresshoff), while indeterminate ones allow the bacterial

population to be established in different longitudinal zones, having simultaneously senescent, fixing and a meristematic developing zone (Vasse *et al*., 1990). Such diversity in nodule phenotypes as well as their role on N fixation will be further explored in Chapter 5.

In terms of the phenotypic expression of the accessions at flowering, it was expected that wilds would have lower values of SDW compared to most of the cultivated accessions as they are inherently smaller plants. The high response in SDW to added N including the wild accessions was also expected, as it is the easiest route for a plant to acquire N; in contrast to the energy costly process of fixing atmospheric N_2 (Postgate 1982). For the wild accessions, differences in SDW between *Rhizobium* and added N treatments were greater than those observed among the cultivated lines with the same treatments (Figure 3.2 A). At flowering, all wild accessions had a tendency to have a smaller biomass under the *Rhizobium* treatment, with values that were not different from the control, but they also had the highest values of N concentration in shoots when inoculated, suggesting a differential mechanism for N fixation between cultivated and wilds accessions (Figure 3.3).

Considering the cost that establishing a symbiotic relationship may represent for the plant (Walley 2013), phenological stages were monitored. With some exceptions in both cultivated and wild accessions, inoculation caused a delay in flowering (Figure 3.1, Table B, Appendix B), compared to +N. Ten out of the 36 accessions studied were not different in flowering time between the two treatments. And, for the sub-group studied up to maturity, there was no difference in DTM between R and +N treatments for most accessions. Only Indianhead and L01-827A had a delay in DTM, of 11.7 and 18.2 days, respectively, and both also experienced a flowering delay. This could have if breeding for better BNF, especially in short season areas, therefore should be taken into consideration and determine the effects of this symbiotic relationship in the phenological cycle under field conditions.

SPAD measurements is often used as a rapid non-destructive measure of leaf chlorophyll content in a range of plant species (Ling *et al*., 2011), however there was a low correlation between N concentration in shoots and SPAD measurements found in this experiment. This could be related to the small, compound, pinnate leaf size, particularly of the wild accessions, which would have interfered with the SPAD readings. Reliable indirect N accumulation measurements need to be

further explored in order to have rapid, cost-efficient and non-destructive measures to accurately estimate N accumulation in lentils, especially for assessing this in larger germplasm collections.

Given the level of specificity and differential interactions that have been demonstrated to occur between *Rhizobium* and various legumes at the accession × strain (Gunnabo *et al*., 2019; Laguerre, *et al*., 2003; Reyes and Planchon 1995), including cultivated lentil (Abi-Ghanem *et al*., 2011), it would be prudent to assess the ability of these *Lens* species with a greater set of strains. Lentil-nodulating *Rhizobium* from all the agroecosystems where the crop is grown have been characterized (Gai *et al*., 2021; Harun-or Rashid *et al*., 2014) and correspond to several *Rhizobium* species. These isolates will also correspond to the centre of origin of lentil and may help confirm the level of specificity of this diverse set to fix N, especially the wilds.

Lens genotypes from no particular species were identified with higher ability to fix N and promising results of seed yield and seed quality traits that can be useful in breeding for better BNF ability. CDC Greenstar was identified as the best resource among the cultivated lines (*L. culinaris*) for its ability to fix high amounts of N and yield similar amounts of seeds when inoculated as when optimal N was added, with no repercussion in seed quality, nor alterations in days to maturity. IG 72643 (*L. orientalis*) and IG 72623 (*L. odemensis*) represent potential resources for increasing N fixing ability, based on seed yield produced with *Rhizobium* inoculation, but also for their unique mechanisms of N use efficiency. Although good resources were identified within the cultivated group, the unique characteristics found in wild accessions suggest that they can play a role in the improvement of the BNF, contributing to +N higher protein content and higher N use efficiency in cultivated lentil.

Prologue to Chapter 4

Chapter 3 was about the effect diverse *Lens* species have in the relationship with rhizobia, based on the interaction with one *Rhizobium* strain - a commercial one. More *Rhizobium* species need to be studied in order to establish the most adequate *Rhizobium* partner for these genotypes. This next chapter focuses on the effect of diverse *Rhizobium* strains on one commercial cultivar.

This chapter will be submitted to Crop Science as soon as our collaborators submitted their manuscript on *Rhizobium* diversity (Gai *et al*., 2021), which was the groundwork for this experiment. Paper and co-author contributions will be as follow:
Vargas, A., Riely, B., Cook, D., Vandenberg, A. and K.E. Bett. 2021. Diverse *Rhizobium* strains differentially affect lentil (*L. culinaris*) performance in the field. Crop Science *in preparation.*

Author contributions:
Vargas, A.: responsible for design, execution, analysis of experiments and preparation of manuscript with input from Bett, K.E.
Riely, B.: selected strains and will revise the manuscript.
Cook, D.: development of initial concepts for the experiments and will review and edit the manuscript.
Vandenberg, A.: development of initial concepts for the experiments and will review and edit the manuscript.
Bett, K.E.: development of initial concepts for the experiments and funding acquisition. Supervised Vargas, A., revised and edited the manuscript.

4. DIVERSE *RHIZOBIUM* STRAINS DIFFERENTIALLY AFFECT LENTIL (*Lens culinaris*) PERFORMANCE IN THE FIELD

4.1. INTRODUCTION

Rhizobia provides nitrogen (N) to many legume crops, diminishing the need for synthetic N fertilizers and minimizing their impact on the environment. Lentils (*Lens culinaris*) fix N in symbiosis with bacteria from the genus *Rhizobium*. *Rhizobium leguminosarum* bv. *viciae* (*Rlv*) is the original symbiont of lentil, which is present in soils where lentil originated and in the areas of later introduction. Other species that infect lentil have been characterized and are endemic to Bangladesh: *Rhizobium lentis*, *Rhizobium bangladeshense* and *Rhizobium binae* (Harun-or Rashid *et al*., 2014). *Rhizobium laguerreae* is the symbiont of lentils in Morocco and nodulates several *Vicia* spp. around the world (Saïdi *et al*., 2014). Several additional species isolated from lentil have recently been characterized (Gai *et al*., 2021).

With crop diversification and shift from a fallow-based system to one that uses legumes on the Canadian Prairies, the soil microbial community was modified (Tanaka *et al*., 2002), and a population of resident rhizobia was established (Lupwayi and Kennedy 2007). Their abundance and effectiveness depend on the cropping history, with larger populations found when peas were previously planted compared to those with a history of wheat (Chemining and Vessey 2005; Kucey and Hynes 1989).

The efficiency of the biological nitrogen fixation (BNF) process has been measured for decades, and estimations of its contributions are well summarized (Unkovich and Pate 2000). The proportion of N derived from the atmosphere (Ndfa) in lentils is around 65%, although a broad range of estimates have been reported from 9 to 97%, resulting in 4 to 152 kg of N ha^{-1} fixed (Peoples *et al*., 2009). In Canada, values of the Ndfa vary from 0 to 87%, with amounts of 0 to 192 kg of N ha^{-1} of total N fixed (Walley, *et al.,* 2007; Bremer *et al*., 1990; Bremer *et al.,* 1988).

With lentils and its rhizobia symbiont coming from a different region in the world, their suitability under a temperate agroecosystem has to be evaluated. Superior *Rhizobium leguminosarum* strains were identified in the past with the varieties Eston and Laird under field conditions in the area of study (Bremer *et al*., 1990). As newer varieties emerge and broader

resources of *Rhizobium* species that can nodulate lentil are identified and become available, local selection with the newest resources has to be conducted.

Selection of diverse sets of strains has delivered suitable inoculum adapted to specific agro-environments (Ruiz-Diez *et al*., 2012a; Ruiz-Diez *et al*., 2012b). Although increases in yield can be observed as a result of the population already established in the soil, more optimum results can be obtained with inoculation. Given the cost efficiency of this practice, producers should always include it in their production program (Vessey 2004). In addition, the inclusion of rhizobia isolated from wild legumes has received more attention for their capacity to infect both wild and cultivated plants across a broad range of adverse conditions (Zahran 2001).

The purpose of this study was to assess the ability to infect and fix N of representatives of the greatest diversity of *Rhizobium* isolated from lentil (both cultivated and wild) currently available. This included six species: *R. leguminosarum*, three species from Bangladesh (*R. lentis*, *R. bangladeshense* and *R. binae*) and two new (yet unnamed species) strains from New Zealand. Such diversity represents a great resource for selection of strains for studying BNF and potential development of inoculants suitable to the Northern Great Plains.

4.2. MATERIALS AND METHODS

4.2.1. *Rhizobium* strains, inoculant production and seed inoculation

The small-seeded (40 g/1000 seeds) red variety CDC Maxim was selected to test the strains, as it is widely grown in the Northern Great Plains and represents the lentil genetics of much of the breeding program in this temperate region.

Thirteen strains of *Rhizobium* were selected from a group representing strains that have been collected from lentil plants in the area of domestication of the crop as well as main production areas (Gai *et al*., 2021) (Table 4.1). Selection was based on a phylogenetic analysis of a global collection isolated from wild and cultivated lentils which fell into 13 major groups. The commercial strain BASF 1435 Nodulator XL© (*Rlv*), commonly used on lentils and peas in western Canada, was also included.

The commercial strain was isolated in Yeast Mannitol Agar (YMA) (YMA, Sigma Aldrich #Y3252) (Table B, Appendix B) from the commercial product and cultured at the same time as the others to standardize all treatments. Cultures were inoculated into sterile Yeast Mannitol

Broth (YMB, Sigma Aldrich #Y3377) and agitated for 2-5 days at 26°C and 180 rpm in a shaker (Orbital, Thermo Scientific, Waltham MA).

Table 4.1. *Rhizobium* strains representing genotypic diversity groups studied under field conditions using the lentil cultivar CDC Maxim for 4 site-years in Saskatchewan, Canada, 2017-2018.

Strain	Species	Origin	Reference
ALM 1	*Rlv*[a]	40°04'00.0" N, 4°50'00.0" W Spain	Ruiz-Diaz 2012
BLR-27 (T)	*R. lentis*	23°03'48.0" N, 88°55'42.0" E Bangladesh	Rashid *et al*. 2014
BLR-195 (T)	*R. binae*	23°03'00.0" N, 91°23'45.0" E Bangladesh	Rashid *et al*. 2014
GLR-13	*Rlv*	49°23'35.9" N, 8°35'39.1" E Germany	Rashid *et al*. 2014
GLR-17	*Rlv*	49°25'00.1" N, 8°43'00.1" E Germany	Rashid *et al*. 2014
GLR-28	*Rlv*	49°57'00.0" N, 8°57'00.0" E Germany	Rashid *et al*. 2014
GLR-54	*Rlv*	48°15'22.2" N, 9°34'51.6" E Germany	Rashid *et al*. 2014
LEN-3	*Rlv*	40°04'00.0" N, 4°26'00.0" W Spain	Ruiz-Diaz 2012
NZLR-1	new species	43°33'31.3" N, 172°02'40.8" E New Zealand	Gai *et al*., 2021
NZLR-24	*Rlv*	43°27'22.3" N, 172°11'25.5" E New Zealand	Gai *et al*., 2021
Oyali B	*Rlv*	37°44'02.6" N, 37°48'11.6" E Turkey	Gai *et al*., 2021
PLR8-1a	*R. bangladeshense*	32°46'50.2" N, 72°41'07.1" E Pakistan	Gai *et al*., 2021
TLR 11	*Rlv*	37°27'40.5" N, 30°03'59.5" E Turkey	Rashid *et al*. 2014
BASF 1435 Nodulator XL©	*Rlv*	BASF Canada[b]	BASF Canada

[a]*Rlv*: *Rhizobium legumisanorum* bv. *viciae.* [b]https://agro.basf.ca/basf_solutions/crops/CON-CIRD-AFY3PQ.

In a preliminary test, strain growth speed was determined based on optical density using a spectrophotometer at 600 nm (ND 1000, Molecular Devices Corp., Sunnyvale, CA). A calibration curve was constructed to ensure the same number of colony-forming units (CFU) for each strain (Figure H, Appendix H). Inoculum was standardized at 1×10^9 cells/ml by density and confirmed by counting cells subsamples stained with fuchsine acid in a Petroff-Hauser chamber under the microscope.

Seeds were inoculated the night before seeding by applying the liquid inoculum mixed with 1% guar gum (w/v) to the surface of the seed and dried overnight. In the morning, seeds were packed in paper envelopes and kept in a cooler on ice until seeding (4-8 hours after). Subsamples of each treatment were taken at the time of planting to monitor inoculum survival, using the most probable number (MPN) serial dilution technique (Somasegaran and Hoben 1994) in Petri dishes with YMA over subsequent days.

4.2.2. Experimental design and statistical analysis

The variety CDC Maxim was tested under field conditions with each of the 13 *Rhizobium* strains, the commercial control and a non-inoculated control for a total of 15 treatments. The experiment was arranged in a Randomized Complete Block Design (RCBD) with eight repetitions. Treatments were distributed in blocks in single plots consisting of nine rows (3.65 m long) with an interrow spacing of 30 cm and a target population of 660 plants per plot.

Each experimental site consisted of 120 plots. A border row of CDC Maxim was planted on both sides of each plot but not inoculated or harvested to establish isolation between plots. An additional separation of 1.5 m in both directions was given between plots.

The experiments were conducted during the normal summer growing seasons at Sutherland (52°09'58.2" N, 106°30'21.8" W) in 2017 and 2018, Rosthern (52°41'21.1" N, 106°18'00.6" W) in 2018 and Clavet (52°04'02.2" N, 106°26'35.3" W) in 2018, all in Saskatchewan, Canada. Soil records from these fields measured from 0-30 cm deep were considered before selecting the environments for the trials. Texture, pH and organic matter content were the main parameters (full soil description-Table I.1, Appendix I). pH values were around 7.0 and organic matter was lower than 3.6 %. Ten soil samples (500 g each) were randomly taken from different parts of the field in each site 1-3 days before planting, to confirm the presence of resident rhizobia using the most probable number (MPN) serial dilution technique (Somasegaran and Hoben, 1994). Crop

management practices were conducted following technical practices used in the area of the study (Table I.2, Appendix I).

A Levene's test was conducted to determine homogeneity of variance among site-years. It was not significant; therefore, treatments were treated as homogeneous and analyzed using a mixed model ANOVA in R (R Core Team, 2018). Site-year was considered as one effect since an unequal number of experiments were conducted in each of 2017 and 2018. Site-years, repetitions within site-years and site-year × strain were considered random effects and strain a fixed effect. Means were separated with the LSD test ($P \leq 0.05$). Results averaged across site-years are presented. Only significant site-year × strain interactions are presented separately or described in the results. A Pearson correlation analysis was also conducted among all measured parameters in R.

4.2.3. Measured parameters

Days to flowering and days to maturity were recorded. Plots were phenotyped at flowering, R1- when 50% of the plants in the field had an open flower. Sutherland 17, Sutherland 18 and Clavet 18 were sampled at 59 days after seeding (DAS) and Rosthern 18 at 61 DAS. Ten whole plants were harvested from the center row of the plot for phenotyping. Manual shovels were used to obtain the plants with the root area, and the size of the shovel (25 cm long) was the reference for the depth to which all root samples were taken. All samples were placed in plastic boxes and stored at 4°C to prevent plants from drying out. The day after, all samples were soaked in water to remove the soil and washed with running water on a metallic mesh to prevent any root loss.

The number of nodules, their location on the roots, and their color were recorded. Location was determined by counting the number of nodules on the 0-15 cm region (NN1) and the 15-25 cm region (NN2). Color was rated based on a 1-3 scale (1: white or green, 2: predominantly pink with some white or green, 3: pink nodules). Shoots were separated from the roots and dried at 70°C for 72 hours to determine shoot dry weight (SDW). Shoots were ground to 1 mm (Cyclone Sample Mill, Seedburo Co., Chicago, IL) and a 250 mg subsample was used to determine N concentration using a LECO analyzer (Leco FP628, Leco Corporation, St. Joseph, MI).

At maturity, when all pods were tan-brown and seed moisture was less than 20 %, another 10 plants from the same row were harvested for estimating thousand seed mass (KSM) and seed percentage protein. Seed percentage protein was determined using LECO analysis on a 250 mg

ground (Cyclone Sample Mill, Seedburo Co., Chicago, IL) seed sample. Rows 2-4 and 6-8 of the plots were bulk harvested mechanically for yield determination. Harvest at Rosthern was 90 DAS, Sutherland 92 DAS both years, and Clavet was at 96 DAS.

4.3. RESULTS

4.3.1. Effects of inoculum strain on biomass and nodulation parameters at flowering

There were significant differences in all biomass and nodulation traits on a site-year basis but there was no significant strain × site-year interaction except for N concentration and nodule colour (Table 4.2). Days to flowering and days to maturity were not differentially affected by strains.

Table 4.2. Shoot dry weight, N concentration in shoots (%) and nodulation parameters of CDC Maxim at flowering (R1), inoculated with one of 14 *Rhizobium* strains or none, averaged across Sutherland, Rosthern and Clavet, SK, Canada, 2017-2018.

			Nodulation parameters			
Source	**Shoot dry weight (g)**	**N concentration in shoots (%)**	**Total number of nodules**	**NN1 0-15 cm**	**NN2 15-25 cm**	**Nodule Colour**
ANOVA[a]						
Strain	ns	***	ns	ns	ns	*
Site-year	***	***	***	***	***	***
Strain × site-year[b]	ns	***	ns	ns	ns	*
Strain						
ALM1	3.0	2.8 abc	53.5	39.6	14.0	2.6abcd
BLR-27 (T)	2.8	2.9 ab	50.7	39.0	11.9	2.7abc
BLR-195 (T)	2.9	2.71 c	50.9	38.7	12.6	2.6abcd
GLR-13	2.8	2.8 bc	56.1	42.3	13.9	2.7abc
GLR-17	2.9	2.7 c	46.6	35.7	10.4	2.6abcd
GLR-28	2.7	2.7 c	47.6	35.3	12.4	2.5cd
GLR-54	3.0	2.7 c	47.3	36.2	11.0	2.7a
LEN-3	2.8	2.9 ab	51.0	37.9	13.1	2.7ab
NZLR-1	2.8	2.9 ab	46.9	35.4	11.4	2.4d
NZLR-24	2.9	2.9 a	44.3	34.5	9.6	2.7abc
Oyali B	2.71	2.74 c	46.3	34.9	11.4	2.6abcd

Table 4.2. Continued.

Source	Shoot dry weight (g)	N concentration in shoots (%)	Nodulation parameters			
			Total number of nodules	NN1 0-15 cm	NN2 15-25 cm	Nodule Colour
PLR8-1a	2.74	2.81 bc	48.3	36.6	11.6	2.6abcd
TLR 11	2.95	2.75 bc	48.5	37.4	11.1	2.6abcd
BASF 1435	2.91	2.80 bc	44.3	33.9	10.6	2.5bcd
Non-inoculated	2.92	2.67 c	51.5	39.0	12.4	2.5cd
LSD $_{(\geq 0.05)}$	ns	0.14	ns	ns	ns	0.21
Site-year						
Sutherland17	1.82d	2.66b	28.6d	19.1d	9.4c	2.4c
Rosthern18	3.15b	2.60b	38.2c	30.8c	7.4d	2.8b
Sutherland18	4.39a	2.76b	73.0a	56.1a	16.9a	2.9a
Clavet 18	2.07c	3.07a	55.9b	42.4b	13.6b	2.3c
LSD $_{(\geq 0.05)}$	0.21	0.14	5.6	4.4	1.8	0.21

[a]ANOVA values are ns: not significant, *, **, ***: significant at 0.05, 0.01, 0.001 respectively. LSD: least significant difference. Means with different letters are significantly different from each other according to LSD test. [b]Significant interactions are described in results.

Shoot Dry Weight (SDW) of CDC Maxim, irrespective of the strain, was the highest in Sutherland 18, with twice the dry biomass accumulated at Sutherland 17 and Clavet 18. The total number of nodules (TNN) was also inconsistent across site-years, with the largest number of nodules observed at those locations with high SDW. The majority of nodules were distributed within the first 15 cm in the soil profile (NN1). No significant differences were found among strains for SDW, TNN, NN1 and NN2 (See Table J.2, Appendix J) for values of this parameters in each site year).

Higher values of N concentration in shoots at flowering were only observed at Clavet 18, with no differences among the other locations (Table 4.2). NZLR-24 (*Rlv*), NZLR1 (new species), ALM1(*Rlv*) and LEN-3 (*Rlv*), were among the strains with higher concentrations of N, contrasting with BLR-195 (T) (*R. binae*), GLR-13 (*Rlv*) and the non-inoculated treatments, which were among the treatments with the lowest values for this trait. A significant interaction was observed for N concentration, in Clavet 18, a value of 3.45 mg g^{-1} with the strain NZLR-24

(*Rlv*) was observed (Table J.1, Appendix J), higher than all other values observed for this trait. BASF 1435 (*Rlv*), NZLR-1 (new species) and the non-inoculated treatment had a significantly higher proportion of yellow/green nodules at this location (Figure 4.1 b).

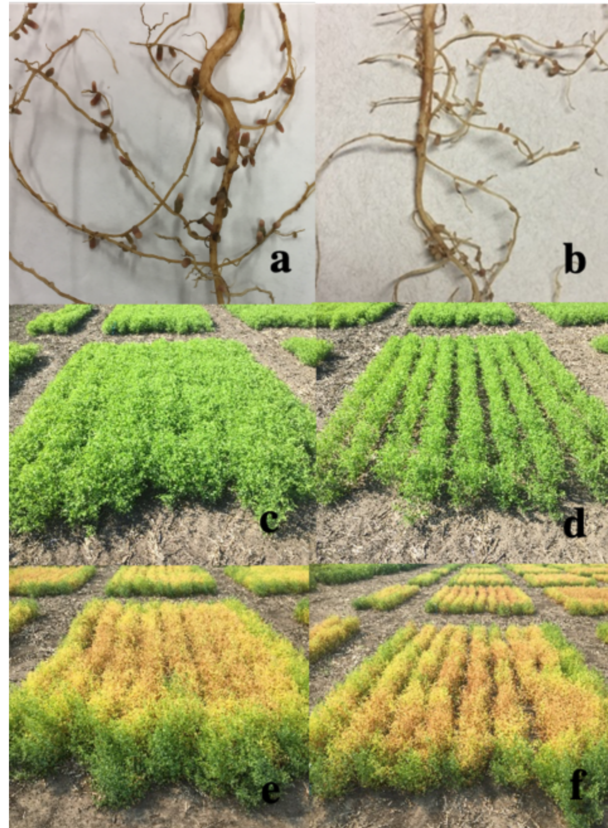

Figure 4.1. CDC Maxim roots at flowering (a and b) following inoculation with the strain NZLR-24 (a, c, e) and non-inoculated (b, d, f). Plots of CDC Maxim 60 DAS (c,d) and 90 DAS (e,f) in Rosthern, SK, Canada, 2018.

4.3.2. Effects of strain on seed traits and yield

A significant effect on yield was observed among strains (Table 4.3). No effects of strain were observed on thousand seed mass (KSM) nor on percentage seed protein. Significant strain × site-year interactions were also observed for those three parameters but only for some specific strains. Seed quality traits were not consistent across all site-years with CDC Maxim (Table 4.3). Seed percentage protein was lower in Rosthern 18 compared to all other site-years. In Sutherland 17, thousand seed mass was higher compared to all other site-years.

Table 4.3. Thousand seed mass (g), seed percentage protein, and yield (kg ha^{-1}) of CDC Maxim, inoculated with one of 14 *Rhizobium* strains or none, averaged across Sutherland, Rosthern and Clavet, SK, Canada, 2017-2018.

Source	Thousand seed mass (g)	Seed percentage protein	Yield (kg ha^{-1})
ANOVA[a]			
Strain	ns	ns	***
Site-year	***	***	***
Strain × site-year[b]	***	**	***
Strain			
ALM1	45.7	24.6	2555.1 def
BLR-27 (T)	45.2	24.9	2580.2 def
BLR-195 (T)	45.1	26.0	2484.8 f
GLR-13	44.4	25.3	2571.8 def
GLR-17	45.3	24.8	2555.0 def
GLR-28	43.9	24.8	2469.0 f
GLR-54	45.0	24.7	2543.1 def
LEN-3	45.8	24.8	2775.0 ab
NZLR-1	44.8	24.4	2638.3 cd
NZLR-24	43.2	24.8	2894.1 a
Oyali B	44.7	24.7	2578.2 def
PLR8-1a	45.2	24.6	2716.1 bc
TLR 11	43.4	24.8	2549.6 def
BASF 1435	43.2	24.6	2612.4 cde
Non-inoculated	44.5	24.8	2507.9 ef
LSD $_{(\geq 0.05)}$	ns	ns	165.32
Siteyear			
Sutherland17	53.6 a	25.5 a	2291.6 bc
Rosthern18	42.6 b	23.6 b	2201.8 c
Sutherland18	41.9 b	25.5 a	2466.6 b
Clavet 18	40.3 c	24.7 a	3448.2 a
LSD $_{(\geq 0.05)}$	2.6	0.8	165.3

[a]ANOVA values are ns: not significant, *, **, ***: significant at 0.05, 0.01, 0.001 respectively. LSD: least significant difference. Means with different letters are significantly different from each other according to LSD test. [b]Significant interactions are presented on a separated table.

From the average values of the strain treatments, plants inoculated with the strain NZLR-24 (*Rlv*) had the highest average yield (Table 4.3), although not significantly higher than with LEN-3 (*Rlv*). The commercial strain BASF 1435 (*Rlv*) yielded significantly lower than NZLR-24 (*Rlv*) and had similar values to those observed on the non-inoculated treatment. An average of 3.4×10^3, 4.2×10^3, 4.1×10^3 and 5.1×10^3cells/ml were observed in Sutherland 17, Rosthern 18, Sutherland 18 and Clavet 18 respectively. Strains from other species BLR-27 (T) (*R. lentis*), BLR-195 (T) (*R. binae*) and PLR8-1a (*R. bangladeshense*) had results similar to those observed with BASF 1435 (*Rlv*). The differences between the strain NZLR-24 (*Rlv*) and those with significantly lower yields, including the non-inoculated plot, were visible in the field plot during early flowering (Figure 4.1 c, d) and also when the plants reached maturity (Figure 4.1 e, f). Different yield values were also observed across site-years. The highest yield was observed in Clavet 18 followed by Sutherland 18, and no differences were observed between Rosthern 18 and Sutherland 17.

Site-year × strain interactions were observed for both protein and yield parameters for some strains. In Sutherland 18, the strains BLR-195 (T) (*R. binae*) and GLR-13 (*Rlv*) had the highest values for percentage seed protein across all experiments (Table 4.4). In Rosthern 18, strain treatments GLR-28 (*Rlv*) and GLR-54 (*Rlv*), yielded the lowest across all site-years across all experiments.

Although LEN-3 (*Rlv*) and PLR8-1a (*R. bangladeshense*) were among the highest-yielding strain treatments on average, both had superior yield values only under specific environments. PLR8-1a had a significant higher yield only in Clavet 18, and LEN-3 (*Rlv*) in Clavet 18 and Rosthern 18. These were not different from the rest of the strains in the other site-years. For KSM, only the strain GLR-17 (*Rlv*) in Sutherland 17 showed a site-year × strain interaction; with the highest value for this trait across the experiments (Table J.3, Appendix J). Sutherland 18 and Rosthern 18 were the site-years with the highest values for nodule colour (NC) (Table 4.2). Across site-years, NZLR 24 (*Rlv*), GLR-54 (*Rlv*) and LEN-3 (*Rlv*), had the highest proportion of pink nodules (NC) (Figure 4.1 a).

Table 4.4. Percentage seed protein and yield (kg ha^{-1}) means of CDC Maxim inoculated with different *Rhizobium* strains that had significant site-year × strain interactions in Sutherland, Rosthern, Clavet, SK, Canada, 2017-18.

	Sutherland 17		**Rosthern 18**		**Sutherland 18**		**Clavet 18**	
Strain	Pct seed protein	Yield (kg ha^{-1})	Pct seed protein	Yield (kg ha^{-1})	Pct seed protein	Yield (kg ha^{-1})	Pct seed protein	Yield (kg ha^{-1})
BLR-195 (T)	25.6	2124.7	23.4	2037.3	**30.8**[a]	2354.3	24.3	3423.0
GLR-13	25.4	2255.5	23.8	2114.0	**27.4**	2529.4	24.8	3388.2
GLR-28	25.8	2152.0	23.7	**1984.8**	25.0	2504.3	24.9	3234.8
GLR-54	25.5	2342.6	23.8	**1960.4**	24.9	2481.7	24.6	3387.5
LEN-3	25.6	2314.5	23.8	**2533.3**	24.9	2533.2	24.8	**3718.5**
PLR8-1a	25.3	2341.2	23.7	2174.4	24.9	2555.9	24.4	**3793.0**
BASF 1435	25.5	2334.2	23.6	2257.3	24.7	2223.4	24.7	3634.8
LSD $_{(\geq 0.05)}$	Protein %= 1.91, Yield= 165.32							

LSD: least significant difference, [a]**bolded** numbers represent the significant interactions.

4.3.3. Correlations among measured parameters

SDW was correlated with TNN and NC, but not with N concentration in shoots nor to any of the parameters measured at maturity (Figure 4.2). N concentration in shoots was highly correlated with yield and also with percentage protein. TNN was highly correlated to percentage protein. NC was not correlated to any of the parameters measured at maturity. Thousand seed mass was not correlated to any other measured parameter.

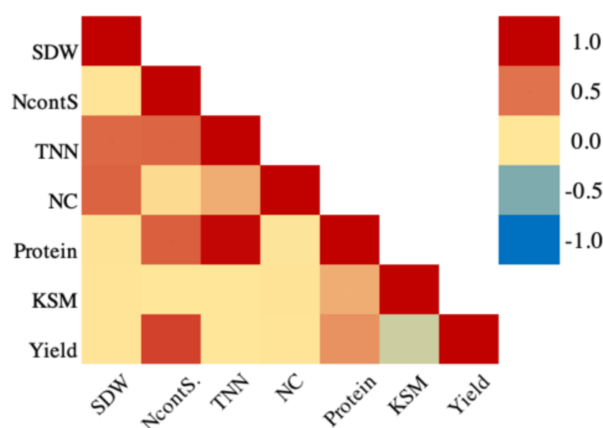

Figure 4.2. Pearson correlations among SDW: shoot dry weight, NcontS: N concentration in shoots, TNN: total nodule number, NC: nodule colour, Protein: seed percentage protein, KSW: thousand seed mass and yield of CDC Maxim with 15 *Rhizobium* treatments across 4 site-years in SK, Canada, 2017-2018.

4.4. DISCUSSION

Exploring a greater diversity of *Rhizobium* strains *in situ* was necessary to understand their contributions to the cropping system under local agro-environmental conditions. These selected set of strains was recently made available and have been genotyped to better understand their relationships (Gai *et al*., 2021). It represents the known diversity of *Rhizobium* nodulating wild and cultivated lentil in their original environment, as well as *Rhizobium* found nodulating cultivated lentil from all production areas in the rest of the world.

Information on the performance of such diverse resources with a newer lentil variety under field conditions was not previously available. Inoculants should be periodically revised, validating their performance with emerging cultivars as well as under changing agro-environmental conditions for their maximum performance (Santos *et al*., 2019; Graham *et al*., 2004). With still inefficient values of Ndfa estimations in the lentil crop in a proportion of the cultivated area in the Northern Great Plains (Peoples *et al*., 2009, Walley *et al*., 2007), improving the main components (lentil and *Rhizobium*) of the BNF relation is essential.

This work shows that there are better resources of strains to improve nitrogen fixation throughout the inclusion of more suitable inoculum. Yield results suggest increases of 15% as an effect of inoculating with the strains NZLR-24 (*Rlv*), compared to non-inoculated plots and 9% when compared to the commercial strain BASF 1435 (*Rlv*). This strain was isolated from lentil plants from a lentil breeding nursery in New Zealand. The similarity in environmental conditions with Saskatchewan - long days and moderate temperatures - during the summer growing season, could be a contributing factor to the favorable response observed. It was tested in one lentil cultivar, CDC Maxim, which is largely cultivated, generated by the Crop Development Centre at the University of Saskatchewan, which is largest breeding program in North America. It will be adequate to evaluate NZLR-24 (*Rlv*) with a greater group of cultivars to validate its range of effectiveness.

The group represented by Oyali B (*Rlv*), that had similar yield results compared to BASF 1435 (*Rlv*), is also of great importance because this group includes strains from the centre of origin and was isolated from wild lentil. Such strains are recognized as an important focus of study for improving N fixation because of their ability to survive under arid conditions, and their capacity to colonize both cultivated and wild plants (Zahran 2001). The inclusion of effective native

rhizobia from America, the centre of origin of climbing beans (*Phaseolus vulgaris*), has been recognized as a determinant of increased yield across variable environment in eastern Africa (Koskey *et al*., 2017). The efficiency of the relationship between this set of *Rhizobium* strains and wild *Lens* species has to be determined.

Yield was not consistent across environments for all top performing strains, making both LEN-3 (*Rlv*) and PLR8-1a (*R. bangladeshense*) less desirable due to their instability. PLR8-1a (*R. bangladeshense*) and LEN-3 (*Rlv*), only had a higher yield in Clavet, SK (Table 4.4). This particular site-year received more precipitation after flowering, which also impacted the overall yield mean for this site. Conversely, NZLR-24 (*Rlv)* showed a consistent response across environments. Thousand seed mass and percentage protein in seeds were not affected by the strain treatments, suggesting that there are no effects of strain on these key grain quality traits and both traits are stable across environments. Additionally, protein was not affected as yield increased - no correlation was observed between these two parameters (Figure 4.2).

From the parameters evaluated at flowering, only N concentration in shoots was correlated with yield. SDW and TNN were not good indirect measures of the effectiveness of the inoculated strains to predict yield, but N in shoot was, similar to what has been reported in previous studies with the lentil cultivars Eston and Laird inoculated with *Rhizobium leguminosarum* strains under field conditions (Bremer *et al*., 1990). There was also a strong correlation between TNN and percentage protein in seeds, similar to what is observed in pea (*Pisum sativum*), where percentage protein is highly correlated to total nodule dry weight (Bourion *et al*., 2007).
It was not totally unexpected that values for TNN were not different among strains, given that they were all tested on one lentil cultivar. The nodule number development process corresponds to systemic phytohormonal signals as well as local signals controlled by the plant genotype (Mortier *et al*., 2012). The variation for this trait among site-years is a response to environmental factors that further influence this trait. Plants do suppress infection threads as a response to specific environmental stresses (Gage 2004) restricting the number of nodules. Any nodules potentially developed below 30 cm were also excluded from evaluation, however the interest was to account only for nodules close to where the inoculant was placed. In addition to this, the biggest proportion of roots was expected to be distributed within that depth. Lentils have shallow root systems, with up to 85% of their roots in the first 40 cm (Gan *et al*., 2009). In different

legumes, including *Lotus japonicus* and barrel clover (*Medicago truncatula*) nodule development is restricted to the specific zone where the primordia are developed (Mortier *et al*., 2012), and the initiation of subsequent nodules is inhibited. As observed in these experiments, most of the nodules were found in the first 15 cm and fewer in the deeper area.

Presence of nodulation in the non-inoculated plot and its similarity with the commercial strain BASF 1435 (*Rlv*) was expected. Fields in this major pulse production area have been exposed to inoculants applied regularly within the last decades and a resident population has been established (Chemining'wa and Vessey 2005). Since there are no indigenous legumes compatible with *Rhizobium legumosarum* bv. *viciae* in the area where the study was conducted, therefore no native population of this bacteria, it is reasonable to assume this population is basically equivalent to commercial products applied over past seasons. Random nodules collected in Saskatchewan from commercial plots corresponded to the groups represented by the GLR-28 (*Rlv*) and GLR-54 (*Rlv*) strains (Gai *et al*., 2021).

The grain yield increases observed thorough inoculation with selected strains is documented in several crops worldwide with increases up to 80% with specific strains in soybean (*Glycine max*) and faba bean (*Vicia faba*) (Ulzen *et al*., 2016, Mercante *et al*., 2017, Youseif *et al*., 2017, Padilla *et al*., 2016). Some of the main approaches proposed to optimize the benefits obtained from rhizobial inoculants in the near future include the expansion of inoculated areas, search for strains that tolerate changing environmental stresses, and inoculants that benefit broader ranges of crops (Santos *et al*., 2019). In the area of this study, for an inoculum to be commercially viable it must not just work with lentil but also with the other cool-season legumes that are found in the same environment. *Rhizobium leguminosarum* bv. *viciae* is the symbiont of faba beans and peas (*Pisum sativum*), which are both economically important in this area (AAFC 2020). Strain NZLR-24 (*Rlv)* was tested for broad use by inoculating pea cv. CDC Amarillo (*Pisum sativum*) and faba bean cv. Snowbird (*Vicia faba*) and growing them in a sterile growth pouch (Figure K, Appendix K). Both peas and faba beans successfully establish symbiosis with this strain. Based on these findings, NZLR-24 (*Rlv*) represents a suitable new option for commercial inoculum production to be used in the pulse cropping system in the Northern Great Plains.

Prologue to Chapter 5

How multiple selected genotypes of *Lens* (Chapter 3) and *Rhizobium* (Chapter 4) interact to influence the efficiency of N fixation will be covered in Chapter 5 by focusing on the effects of the specificity using accessions from 5 *Lens* species × 3 *Rhizobium* species.

This chapter will be submitted to Crop Science. Paper and co-author contributions will be as follow:

Vargas, A. and K.E. Bett. 2021. Symbiosis of *Lens* species with diverse *Rhizobium* strains: effects of specificity on their biological nitrogen fixation ability. Crop Science *in preparation.*

Author contributions:

Vargas, A.: responsible for designing concepts, execution, analysis of experiments and preparation of manuscript with input from Bett, K.E.

Bett, K.E.: funding acquisition, supervised Vargas, A., revising and editing the manuscript.

5. SYMBIOSIS OF *LENS* SPECIES WITH DIVERSE *RHIZOBIUM* STRAINS: EFFECTS OF SPECIFICITY ON THEIR BIOLOGICAL NITROGEN FIXATION ABILITY

5.1. INTRODUCTION

The relationship between N fixing bacteria and legume crops, has played a key role in the use of atmospheric N to fertilize all major legume crops grown in the world (Herridge *et al*., 2008). Nod Factor (NF) receptors in legumes hosts evolved to allow highly specific recognition of NF, crucial for more efficient specific symbiotic systems (Remigi *et al*., 2016, Bisseling and Geurts 2020).

Selection for enhanced nitrogen fixation in lentil (*Lens culinaris*) has focused on the cultivated pool (Abi-Ghanem *et al*., 2011; Hafeez *et al*., 2000), but limited breeding has occurred. Wild relatives play a role in breeding for biotic and abiotic factors (Muñoz *et al*., 2017). There are six wild relative species in the genus *Lens* (Wong *et al*., 2015) and several have been identified as good sources of disease resistance, such as anthracnose, ascochyta blight and stemphylium blight (Vail *et al*., 2012; Podder *et al*., 2012; Gupta and Sharma 2006). Differential N fixing ability among accessions from these species has been established (Chapter 3).

There are several species of *Rhizobium* that nodulate *Lens* species (Gai *et al.*, 2021). *Rhizobium leguminosarum* bv. *viciae* is the original symbiont of lentil, but others, including *R. lentis*, *R. bangladeshense*, *R. binae* and *R. laguerreae*, correspond to specific adaptation events in symbiosis with locally adapted lentils (Saïdi *et al*., 2014; Harun-or-Rashid *et al*., 2012).

The role of specificity at the strain × accession level has proven to be important to the effectiveness of this relationship in a number of legume crops. Both the strain and legume genotype determine the N fixation effectiveness of this symbiotic relationship at different levels. In some cases, it can be very specific, such as in pea (*Pisum sativum*) (Yang *et al*., 2017), common bean (*Phaseolus vulgaris*) (Gunnabo *et al*., 2019; Valberde and Ottabong 1997), barrelclover (*Medicago trunculata*) (Bena *et al*., 2005), soybean (*Glycine max*) (Ramongolalaina *et al*., 2018; Sugiyama *et al*., 2015) and lentil (Abi-Ghanem *et al*., 2011). In other cases, it can have only moderate specificity, as is the case of tepary bean (*Phaseolus acutifolius*) (Somasegaran and Hoben 1991). Very promiscuous associations have been identified, mostly in tropical legumes such as *Arachis* species (Somasegaran and Hoben 1994).

The symbiotic process occurs in specialized structures called nodules. Strain-specific control of effective fixation in nodules is determined by the *MtNFS1* and *MtNFS2* genes in medicago (Wang *et al*., 2018; Wang *et al*., 2017., Yang *et al*., 2017), and by the *APN1* gene in *Lotus japonicus* (Yamaya-Ito *et al*., 2018).

There are determinate and indeterminate nodules (Sprent *et al*., 2013). Determinate nodules have a globose shape and the members of the bacteroid population reach senescence at the same time (Rolfe and Gresshoff 1988). Indeterminate nodules have an elongated shape and the bacteroids are organized into differentiated zones: an active meristem, the infection zone, as well as nitrogen-fixing and senescent zones (Vasse *et al*., 1990), that can result in bifurcate, palmate or coralloid structures. Differences between determinate and indeterminate nodules have been attributed to a *MtNFH1* Nod Factor (NF) mutation in medicago, with different phenotypes of indeterminate nodules in the presence of reduced *MtNFH1* activity with the strain *Sinorhizobium meliloti* 1021 (Cai *et al*., 2018). Species that can produce both determinate and indeterminate types are uncommon (Fernandez-Lopez *et al*., 1998; Liu *et al*., 2014). *Lens* species, however, do produce both types of nodules (Zahran, *et al*., 2013; Riah *et al*., 2014; Chapter 3).

Although it is known that lentil exhibits a level of specificity in associations with *Rhizobium leguminosarum* bv. *viciae* (Hafeez *et al*., 2000), observations with a broader set of *Lens* species × *Rhizobium* species are not available. With more diverse germplasm being used in the breeding program, it is necessary to understand how this inclusion of greater diversity will impact the efficiency of symbiotic relations in the lentil crop.

The objective of this study was to characterize N fixation related traits of specific relationships between selected groups of *Lens* species (Chapter 3) and *Rhizobium* (Chapter 4) to determine the role of the accession × strain effect on the effectiveness of this relation and its implications for the breeding of higher biological nitrogen fixation (BNF) ability in lentils.

5.2. MATERIALS AND METHODS

5.2.1. Accessions and *Rhizobium* strains

The 10 *Lens* accessions from 6 species, and 5 *Rhizobium* strains from 3 species, that were tested are listed in table 5.1. The accessions were selected based on their differential N fixing ability

established in Chapter 3. The *Rhizobium* strains were selected for their diversity and performance under field conditions studied in Chapter 4.

Table 5.1. Accession, gene pool, species and origin of 10 *Lens* species accessions and the five *Rhizobium* strain treatments inoculated.

***Lens* accessions**			
Accession	Species	Gene Pool	Country of origin
CDC Greenstar	*L. culinaris*	Primary	Canada[a]
Eston	*L. culinaris*	Primary	Canada
VIR-421	*L. culinaris*	Primary	Italy
Lupa	*L. culinaris*	Primary	Spain
BGE 016880	*L. orientalis*	Primary	Israel
IG 72643	*L. orientalis*	Primary	Syria
PI 572390	*L. tomentosus*	Secondary	Turkey
IG 72623	*L. odemensis*	Secondary	Turkey
IG 110810	*L. lamottei*	Secondary	Spain
L01-827A	*L. ervoides*	Tertiary	Unknown[b]
***Rhizobium* strains**			
Strain	Species	Reference	Country of origin
BLR-27 (T)	*Rhizobium lentis*	Rashid *et al*. 2014	Bangladesh
PLR8-1a	*Rhizobium bangladeshense*	Rashid *et al*. 2014	Pakistan
NZLR-24	*Rlv*[d]	Gai *et al*., 2021	New Zealand
Oyali B	*Rlv*	Gai *et al*., 2021	Turkey
BASF 1435	*Rlv*	BASF Canada[c]	Canada

[a]Crop Development Centre, University of Saskatchewan SK Canada. [b]Fiala *et al*., 2009. [c]https://agro.basf.ca/basf_solutions/crops/CON-CIRD-AFY3PQ. [d]*Rlv*: *Rhizobium leguminosarum* bv. *viciae.* White: cultivated and grey: wild accessions.

5.2.2. Experimental design and statistical analysis

Treatments were arranged in a split-plot design (to prevent cross contamination among strains and to control plots), with each pot representing an experimental unit. Treatments were the main plots, including 5 *Rhizobium* strains and a non-inoculated control with added N (+N) for a total of 6 treatments. The 10 lentil accessions were randomized to the subplots with 8 replicates each for a total of 480 experimental units and the whole experiment was repeated twice. Sets of 4 replicates were phenotyped at 40 days after sowing (DAS) and the other 4 at maturity. For the statistical analysis an ANOVA test was conducted with main plots and accessions considered as

fixed and replicates as random, to test for significance of effects. Means were separated using a least significant difference test (LSD; $P \leq 0.05$) using the statistical package SAS (SAS Institute, 2015). A Pearson correlation matrix was also generated among means of all measured parameters in SAS.

5.2.3. Growing conditions and inoculum.

The experiments were conducted in a growth chamber in the phytotron facility in the Agriculture Building, U of S during 2019-2020. The chamber was set to a 16-hour day length and temperature was set to 21 ˚C during the day and 15 ˚C at night. Seeds were disinfected, scarified and pre-germinated in soft agar (6% w/v) 48-72 hours before planting. For disinfection, seeds were surface sterilized with 70% ethanol (v/v) for 30 seconds, followed by 5% bleach (v/v) for 2 minutes, and washed with running distilled water. Scarification was conducted to ensure germination of the wild accessions and carried out manually by nicking the seed coat with a razor blade before plating. A N-free growing mix composed of vermiculate and sand (1:1 v/v) was used to ensure accurate estimation of N fixed. Media was autoclaved for 30 minutes at 121 ˚C and 15 psi to ensure the absence of *Rhizobium* and other microbial populations. N-free nutrient solution (Broughton and Dilworth, 1970, Table M, Appendix M) was applied to growing media for the first time 24 hours before planting the germinated seeds in 1-gallon pots (6 1/2 "diameter). Nutrient solution was applied every 5 days until plants were sampled. KNO_3 was used in the +N treatment at a rate of 0.5 g/L applied at the same time as the N-free nutrient solution. The same amount of water or nutrient solution was applied to each plant regardless of treatment as follows: from 0-7 days after sowing (DAS), 300 ml total; from 8-14 days, 100 ml daily; from 14-40 days, 150 ml daily; and from 41 to maturity, 300 ml daily.

Pure *Rhizobium* cultures were grown on yeast mannitol agar (YMA, Sigma Aldrich #Y3252) (Table B, Appendix B) and transferred after 48 hours to yeast mannitol broth (YMB, Sigma Aldrich #Y3377). The liquid culture was agitated in a shaker (Orbital, Thermo Scientific, Waltham MA) for 48 hours at 26℃ and 180 rpm. Liquid inoculum was standardized to 3×10^9 cells/ml and inoculation was conducted 24 hours after transferring the germinated seeds to the growing medium by applying 1 ml of liquid inoculum directly to the germinated seedling. Inoculation was repeated 48 hours later to ensure infection.

5.2.4. Parameters phenotyped

5.2.4.1. At flowering

Days to flower (DTF) was recorded when the first flower opened. Four replicates were sampled. At 40 DAS, nodulation, root and shoot phenotyping was conducted on these replicates. Shoots were separated from roots using scissors and dried at 70℃ for 72 hours to determine shoot dry weight (SDW-g). Roots were washed with cold water using a 5 mm screen separating all growing media from the roots while keeping the entire root system. Washed roots were placed in Ziploc® bags in a fridge at 6℃ to prevent dehydration until they were evaluated. Presence of nodulation was scored by counting the nodule number (NN) and nodule dry weight (NDW-mg) was estimated following desiccation at 60℃ for 48 hours. Specific nodule dry weight (SpNDW) was estimated by dividing NDW by NN. Nodule fresh weight (NFW) was also recorded (Table N, Appendix N) but not used in the main analysis. Since all samples were stored in sealed plastic bags after being sampled, fresh weights are accurate. Pictures were taken of nodule phenotypes. Roots were then dried at 70℃ for 72 hours and root dry weight (RDW-g) was measured. Root to shoot ratio (R:S) was calculated using RDW and SDW. A shoot and a root subsample were grinded (Cyclone Sample Mill, Seedburo Co., Chicago, IL) to determine shoot and root N concentration on a 100 mg sample using a LECO analyzer (Leco FP628, Leco Corporation, St. Joseph, MI). N accumulation was estimated as a product of N concentration in shoots and roots and RDW and SDW. These values were used to determine the N fixed by using the N balance method (Equation 5.1; Unkovich *et al*., 2008). N fixation efficiency of nodulation was calculated in terms of N_2 fixed/ dry nodule weight. Since no N was present in the growing media of the inoculated treatments, any N obtained by the plant was obtained from fixation. The N present in the sown seed was previously estimated in a 50 seed subsample from the same source as the sown seed, on 100 mg samples using a LECO analyzer (LECO FP628, Leco Corporation, Saint Joseph, MI).

$$N_2 \text{ fixed (N balance method)} = [\text{N yield shoots } (N/100*SDW) + \text{N yield roots } (N/100*RDW)] - \text{N in sown seed} \quad \text{Equation (5.1)}$$

5.2.4.2. At maturity

Days to maturity (DTM) were recorded for each entry when about 90% of the pods were brown and seed moisture less than 20%. Four replicates were sampled. Whole plants were harvested

and dried at 50℃ for 72 hours. Seeds were separated, counted (SN) and weighed to determine total seed weight per plant (SW-g) and the rest of the plant was weighed and added to the SW-g to obtain plant dry weight. Harvest index (HI) was estimated as (SW/whole plant dry weight) × 100. Seed sub-samples (approx. 30-50 units) were ground to 2 mm with a seed grinder (Cyclone Sample Mill, Seedburo Co., Chicago, IL). Seed percentage protein was determined on 100 mg as described in 5.2.4.1. Protein yield (PY) was estimated as (seed percentage protein × SW)/100. Thousand seed weight (KSW) was estimated as (SW/SN) × 1000.

5.3. RESULTS

5.3.1. N fixation, nodulation and nodulation efficiency at flowering.

The effect of strain was significant for all parameters except for specific nodule dry weight (SpNDW), and differences among accessions as well as accession × strain interactions were observed for all parameters (Table O, Appendix O for ANOVA). Most of the N accumulated was allocated in the shoots and only a small proportion was obtained from roots for all accessions under all strains and +N treatments (Figure P, Appendix P, Table Q, Appendix Q).

Different amounts of N fixed were observed among strains and accessions, with all values being significantly lower than the N accumulated in the +N treatment at flowering (Table 5.2). NZLR-24 (*Rlv*) had superior values of N fixed compared to all the other strains, including BASF 1435 (*Rlv*), followed by Oyali B (*Rlv*). Both BLR-27 (T) (*R. lentis*) and PLR8-1a (*R. bangladeshense*) had the lowest values for N fixation. Among accessions, when inoculated with NZLR-24 (*Rlv*), CDC Greenstar accumulated twice the amount observed as when inoculated with BASF 1435 (*Rlv*) and this was the only accession × strain combination to have a significantly higher value than the +N control. Eston fixed the most N with NZLR-24 (*Rlv*). Both VIR-421 and Lupa had N fixing values that were lower than Greenstar and Eston, and similar to the values observed for all the wilds. BGE 016880 (*L. orientalis*) and IG 72623 (*L. odemensis*) only had lower N fixed values with PLR8-1a (*R. bangladeshense*) compared to the rest of treatments. IG 72643 (*L. orientalis*) had higher values with strain Oyali B (*Rlv*) and L01-827A (*L. ervoides*) with BASF 1435 (*Rlv*).

Table 5.2. N fixed (mg/plant) estimated by the balance method and efficiency of nodulation (N_2 fixed/dry nodule weight) of 10 *Lens* species accessions inoculated with one of five *Rhizobium* strains or added N treatments (mean ± SD).

Accession	**BLR-27 (T)**		**PLR8-1a**		**NZLR-24**	
	N fixed (mg/plant)[a]	**Efficiency**[b]	**N fixed (mg/plant)**	**Efficiency**	**N fixed (mg/plant)**	**Efficiency**
CDC Greenstar	24.4 (±3.2)	38.1 (±14.8)	100.1 (±19.3)	229.9 (±33.5)	**479.1** (±56.1)	447.4 (±31.3)
Eston	37.8 (±5.5)	54.2 (±5.5)	42.1 (±12.8)	38.8 (±6.5)	241.3 (±22.7)	393.6 (±18.5)
Lupa	1.7 (±1.0)	9.8 (±4.1)	6.3 (±2.0)	37.7 (±9.9)	42.5 (±14.0)	122.6 (±22.1)
VIR-421	20.2 (±3.7)	31.1 (±4.6)	10.5 (±2.9)	15.9 (±4.0)	44.9 (±18.2)	52 (±8.4)
BGE 016880	27.3 (±4.6)	136.5 (±13.2)	0 (±0)	0 (±0)	45.6 (±6.5)	159.7 (±19.6)
IG 72643	7.2 (±2.3)	38.6 (±4.8)	4.4 (±1.3)	26.7 (±5.6)	8.03 (±1.5)	65.2 (±15.5)
IG 72623	27.4 (±4.6)	92 (±9.7)	0.9 (±0.4)	11.3 (±3.4)	46.9 (±6.5)	239.5 (±19.8)
PI 572390	11.4 (±2.1)	61.3 (±5.9)	17.5 (±3.6)	**615.5** (±85.8)	27.6 (±2.7)	87.4 (±4.2)
IG 110810	0 (±0)	0 (±0)	0 (±0)	0 (±0)	26.6 (±01.8)	104.7 (±8.0)
L01-827A	0 (±0)	0 (±0)	0 (±0)	0 (±0)	9.3 (±1.3)	118.4 (±38.2)
Mean	15.8	46.2	18.2	97.6	97.2	179
LSD	N fixed= 12.9***, efficiency= 37.9***					

Table 5.2. Continued.

Accession	Oyali B		BASF 1435		+Nitrogen[c]
	N fixed (mg/plant)	**Efficiency**	**N fixed (mg/plant)**	**Efficiency**	**N accumulated**
CDC Greenstar	193.1 (±27.5)	536.9 (±42.6)	228.9 (±22.1)	772.5 (±32.7)	467.3 (±35.2)
Eston	60.3 (±6.7)	68.1 (±5.5)	185.1 (±14.6)	441.8 (±8.7)	487.1 (±38.6)
Lupa	9.4 (±1.9)	72.9 (±13.3)	12.1 (±2.8)	126 (±17.9)	126.4 (±17.7)
VIR-421	3.2 (±1.3)	16.6 (±1.7)	35.0 (±4.6)	146.2 (±9.4)	387.6 (±30.0)
BGE 016880	18.7 (±2.0)	108.3 (±4.3)	36.1 (±3.3)	105.3 (±9.2)	149.0 (±11.8)
IG 72643	29.1 (±4.1)	85.6 (±7.0)	19.6 (±3.1)	146.5 (±15.8)	254.7 (±22.0)
IG 72623	32.4 (±3.7)	227.9 (±34.3)	18.2 (±3.8)	132.6 (±16.1)	289.2 (±26.0)
PI 572390	37.4 (±4.1)	90.5 (±6.2)	39.4 (±4.1)	138 (±7.2)	268.0 (±24.1)
IG 110810	15 (±1.2)	62.2 (±3.4)	19.1 (±1.3)	98.2 (±8.5)	234.3 (±18.2)
L01-827A	5.4 (±1.7)	56.7 (±11.8)	14.1 (±1.9)	113.7 (±6.2)	160.2 (±17.8)
Mean	40.4	132.6	60.8	222.1	282.2
LSD	N fixed= 12.9***, efficiency= 37.9***				

[a]N_2 fixed (N balance method) = [N yield shoots (N/100*SDW) + N yield roots (N/100*RDW)]-N in sown seed, [b]Efficiency= (N_2 fixed/dry nodule weight), ANOVA values are ns: not significant, *, **, ***: significant at 0.05, 0.01, 0.001 respectively. [c]Added chemical N fertilizer. **Bolded** numbers represent the significant interactions for accession × strain. White: cultivated and grey: wild accessions.

The percentage values for efficiency of nodulation (Table 5.2), varied among all strains. For this measurement, BASF 1435 (*Rlv*) has the highest overall values, followed by NZLR-24 (*Rlv*) and Oyali B (*Rlv*). BLR-27 (T) (*R. lentis*) and PLR8-1a (*R. bangladeshense*) had the lowest efficiency of nodulation observed.

Among the cultivated accessions, CDC Greenstar had similar values of efficiency with NZLR-24 (*Rlv*) compared to BASF 1435 (*Rlv*). Eston, Lupa and VIR-421 had lower nodulation efficiency with all strains as compared to BASF 1435 (*Rlv*). Among the wild accessions, BGE 016880 (*L. orientalis*), had the highest nodulation efficiency with NZLR-24 (*Rlv*), and values with BLR-27 (T) (*R. lentis*) and Oyali B (*Rlv*) that were not different from BASF 1435 (*Rlv*). IG 72623 (*L. odemensis*) had more efficient nodulation with the strains NZLR-24 (*Rlv*) and Oyali B (*Rlv*). PI 572390 (*L. tomentosus*) had the most efficient nodulation values with the strain PLR8-1a (*R. bangladeshense*). IG 110810 (*L. lamottei*) and L01-827A (*L. ervoides*) did not fix any N with the strains BLR-27 (T) (*R. lentis*) and PLR8-1a (*R. bangladeshense*).

There was variation in the average number of nodules (NN) observed among strains and accesions within strains at flowering (Table 5.3). Accessions inoculated with the strains with the lowest N fixing strains BLR-27 (T) (*R. lentis*) or PLR8-1a (*R. bangladeshense*), had the largest NN, in contrast to those inoculated with NZLR-24 (*Rlv*) or BASF 1435 (*Rlv*) which had the lowest NN and the highest amount of N fixed at flowering.

Among the cultivated accessions, CDC Greenstar, Eston and VIR-421 exhibit the largest NN with differences when inoculated with different strains. Lupa had higher NN values with the strain PLR8-1a (*R. bangladeshense*), and lower values with the rest of the strains, similar to those observed on the wild accessions. IG 72623 (*L. odemensis*) showed higher NN values with the strains BLR-27 (T) (*R. lentis*) and NZLR-24 (*Rlv*) similar those observed in the cultivated ones, but not with the rest of strains. The rest of the wild accessions varied in their NN values under all strains but were always lower than the cultivated ones. L01-827A (*L. ervoides*) was the accession with the lowest NN observed with all strains.

Table 5.3. Number of nodules, nodule dry weight (mg) and specific nodule dry weight (mg) of 10 *Lens* species accessions inoculated with one of five *Rhizobium* strains

Accessions	**BLR-27 (T)**			**PLR8-1a**			**NZLR-24**	
	NN	**NDW**	**SpNDW**[a]	**NN**	**NDW**	**SpNDW**	**NN**	**NDW**
CDC Greenstar	413.7 (±9.3)	63.5 (±1.9)	0.2 (±0.01)	152.7 (±1.3)	43.3 (±2.8)	0.3 (±0.02)	315.2 (±8.2)	**108.0 (±16.9)**
Eston	236.4 (±5.2)	69.4 (±2.1)	0.3 (±0.01)	492.5 (±10.1)	**108.7 (±6.9)**	0.2 (±0.01)	197 (±2.1)	61.2 (±1.9)
Lupa	78.8 (±1.6)	16.6 (±2.1)	0.2 (±0.01)	**118.2** (±3.5)	16.4 (±2.5)	0.1 (±0.01)	83.7 (±1.9)	49.0 (±7.5)
VIR-421	236.4 (±5.1)	64.6 (±2.1)	0.3 (±0.01)	344.8 (±8.5)	65.6 (±2.1)	0.2 (±0.01)	187.2 (±3.9)	53.9 (±3.4)
BGE 016880	27.6 (±0.6)	19.9 (±1.9)	0.7 (±0.05)	29.6 (±0.8)	2.1 (±0.4)	0.1 (±0.07)	19.7 (±0.5)	28.5 (±1.0)
IG 72643	49.3 (±1.3)	18.3 (±2.0)	0.4 (±0.03)	88.7 (±2.0)	16.4 (±2.6)	0.2 (±0.01)	17.7 (±0.5)	15.1 (±6.5)
IG 72623	**177.3** (±4.1)	29.7 (±2.9)	0.2 (±0.01)	59.1 (±1.7)	7.7 (±1.2)	0.1 (±0.01)	118.2 (±3.3)	19.5 (±1.1)
PI 572390	57.1 (±1.5)	18.6 (±2.2)	0.3 (±0.1)	29.6 (±0.9)	6.0 (±0.1)	0.2 (±0.06)	78.8 (±1.7)	31.6 (±3.3)
IG 110810	24.6 (±0.5)	33 (±16.2)	1.3 (±0.08)	18.7 (±0.5)	4.0 (±0.2)	0.2 (±0.01)	39.4 (±1.1)	25.3 (±1.9)
L01-827A	14.8 (±0.3)	1.5 (±0.1)	0.1 (±0.01)	13.8 (±0.5)	28.4 (±14.3)	**2.1** (±0.3)	12.8 (±0.5)	10.1 (±0.8)
Mean	131.6	33.5	0.4	134.8	30.1	0.4	107.0	40.2
LSD	Number of nodules= 3.9^{***}, nodule dry weight= 10.85^{***}, specific nodule weight= $0.29^{***§}$							

Table 5.3. Continued.

Accessions	**NZLR-24**	**Oyali B**			**BASF 1435**		
	SpNDW	**NN**	**NDW**	**SpNDW**	**NN**	**NDW**	**SpNDW**
CDC Greenstar	0.3 (±0.1)	246.3 (±5.4)	35.84 (±2.8)	0.2 (±0.01)	197 (±4.6)	29.6 (±1.7)	0.2 (±0.01)
Eston	0.3 (±0.1)	374.3 (±8.2)	88.29 (±2.8)	0.2 (±0.01)	177.3 (±4.3)	41.86 (±2.9)	0.2 (±0.02)
Lupa	0.6 (±0.1)	59.1 (±1.8)	13.1 (±1.3)	0.2 (±0.01)	49.3 (±1.7)	9.5 (±0.9)	0.2 (±0.01)
VIR-421	0.3 (±0.01)	177.3 (±4.4)	19 (±2.9)	0.1 (±0.01)	118.2 (±3.4)	23.9 (±2.1)	0.2 (±0.01)
BGE 016880	1.5 (±0.04)	29.6 (±0.7)	17.2 (±2.7)	0.6 (±0.09)	34.5 (±1.1)	34.23 (±2.4)	1.0 (±0.05)
IG 72643	0.9 (±0.06)	78.8 (±1.8)	33.84 (±2.5)	0.4 (±0.02)	25.6 (±0.5)	13.6 (±2.1)	0.5 (±0.05)
IG 72623	0.2 (±0.03)	70.9 (±1.6)	14.2 (±0.8)	0.2 (±0.01)	39.4 (±1.0)	13.8 (±2.7)	0.4 (±0.05)
PI 572390	0.4 (±0.03)	93.6 (±1.9)	41.18 (±2.0)	0.4 (±0.03)	73.9 (±1.7)	28.5 (±2.1)	0.4 (±0.05)
IG 110810	0.6 (±0.04)	19.7 (±0.6)	24 (±1.8)	1.2 (±0.1)	27.6 (±0.9)	19.3 (±1.9)	0.7 (±0.05)
L01-827A	0.8 (±0.05)	23.6 (±0.5)	9.5 (±1.1)	0.4 (±0.08)	5.9 (±0.3)	12.4 (±1.7)	**2.1** (±0.4)
Mean	0.6	117.3	29.6	0.4	74.9	22.7	0.6
LSD	Number of nodules= 3.9***, nodule dry weight= 10.85***, specific nodule weight= 0.65***						

[a]Specific nodule weight: NDW/NN, ANOVA values are ns: not significant, *, **, ***: significant at 0.05, 0.01, 0.001 respectively. **Bolded** numbers represent the significant interactions for accession × strain. White: cultivated and grey: wild accessions.

The treatment mean for nodule dry weight (NDW) was only higher with the strain NZLR-24 (*Rlv*) when compared to BASF 1435 (*Rlv*), but not different from the rest of the strains (Table 5.3). CDC Greenstar had the highest NDW when inoculated with the strain NZLR-24 (*Rlv*) and Eston with the strain PLR8-1a (*R. bangladeshense*). The average mean for specific nodule dry weight (SpNDW) was not different among strains (Table 5.3). Only some wild accessions varied in their SpNDW, but none of the cultivated ones did. L01-827A (*L. ervoides*) had the highest SpNDW observed in the experiment with the strains PLR8-1a (*R. bangladeshense*) and BASF 1435 (*Rlv*). BGE 016880 (*L. orientalis*) presented higher values of SpNDW with the strains NZLR-24 (*Rlv*) and BASF 1435 (*Rlv*) and IG 110810 (*L. lamottei*) with BLR-27 (T) (*R. lentis*) and Oyali B (*Rlv*).

5.3.2. Nodule phenotypes.

Nodule phenotypes varied among accessions and at the accession × strain level in some cases. Phenotypes presented in Figure 5.1 are the predominant and distinctive phenotypes for each accession × strain combination. The full phenotypic nodulation distribution in a single plant is available in Figure R, Appendix R.

The four cultivated accessions had mainly determinate, unbranched, nodule phenotypes, and the difference observed was in how globose or elongated they were. CDC Greenstar × BASF 1435 (*Rlv*) was of a globose type, while Lupa × BASF 1435 (*Rlv*) was one of the more elongated types. A small proportion of their nodules were indeterminate bifurcate.

Wild accessions exhibited both determinate and indeterminate nodule phenotypes, with a bigger proportion of indeterminate nodules compared to cultivars and more diverse indeterminate types. BGE 016880 (*L. orientalis*) had indeterminate nodules with all the strains but with differing structures based on the specific strain: bifurcate with NZLR-24 (*Rlv*) and BASF 1435 (*Rlv*), palmate with PLR8-1a (*R. bangladeshense*) and Oyali B (*Rlv*), and palmate-collaroid with Oyali B (*Rlv*). Similarly, IG 110810 (*L. lamottei*) and L01-827A (*L. ervoides*) showed combinations of indeterminate phenotypes depending on the strains.

Figure 5.1. Nodule phenotypes of ten *Lens* spp. accessions inoculated with one of five *Rhizobium* strains from 3 species photographed 40 days after seeding. A: determinate, B, C, D: indeterminate of types B: bifurcate, C: palmate and D: collaroid. Bars: 0.75 cm. White: cultivated and grey: wild accessions.

IG 72643 (*L. orientalis*) had determinate, globose nodules only with the strain PLR8-1a (*R. bangladeshense*) and diverse combinations of indeterminate types with the rest of the strains. IG 72623 (*L. odemensis*) exhibited determinate nodules with PLR8-1a (*R. bangladeshense*), Oyali B (*Rlv*) and BASF 1435 (*Rlv*), while PI 572390 (*L. tomentosus*) only had determinate nodules with Oyali B (*Rlv*). All photographs were taken at 40 days, so some indeterminate nodules were starting to present a senescent appearance, mainly characterized by a darker pink or brownish color such as observed in BLR-27 (T) (*R. lentis*) × BGE 016880 (*L. orientalis*).

5.3.3. Effect of strains on yield and seed traits.

Differences among the strain treatments, accessions and accession × strain interactions were observed for all the seed parameters measured at maturity. See table S.1 and S.2, Appendix S for all means of plots presented in section 5.3.3 and table O, Appendix O for ANOVA. The seed number (SN) per plant varied depending on the combination of strain and genotypes (Figure 5.2 A). Among strains, SN values were the highest when inoculated with NZLR-24 (*Rlv*) or BASF 1435 (*Rlv*), followed by Oyali B (*Rlv*) and BLR-27 (T) (*R. lentis*), and the lowest were with PLR8-1a (*R. bangladeshense*). All inoculated treatments had significantly lower SN compared to +N treatment (Figure 5.2 A).

Among the cultivated accessions, only CDC Greenstar, when inoculated with NZLR-24 (*Rlv*), had larger SN compared to the +N treatment and inoculation with BASF 1435 (*Rlv*). Lupa had values similar to the +N only when inoculated with NZLR-24 (*Rlv*). Two wild accessions showed differences between inoculations with NZLR-24 (*Rlv*) or BASF 1435 (*Rlv*): IG 72623 (*L. odemensis*) had the highest SN with NZLR-24 (*Rlv*), and L01-827A (*L. ervoides*) with BASF 1435 (*Rlv*). At the same time, IG 72623 (*L. odemensis*) was the only accession that had a similar SN when inoculated with BLR-27 (T) (*R. lentis*), compared to the rest of strains. When inoculated with the strain Oyali B (*Rlv*), most wild accessions, except L01-827A (*L. ervoides*) and IG 72623 (*L. odemensis*), had SN values that were not different from those observed when inoculated with BASF 1435 (*Rlv*) or NZLR-24 (*Rlv*). CDC Greenstar did not differ in SN when inoculated with Oyali B (*Rlv*) or BASF 1435 (*Rlv*) either.

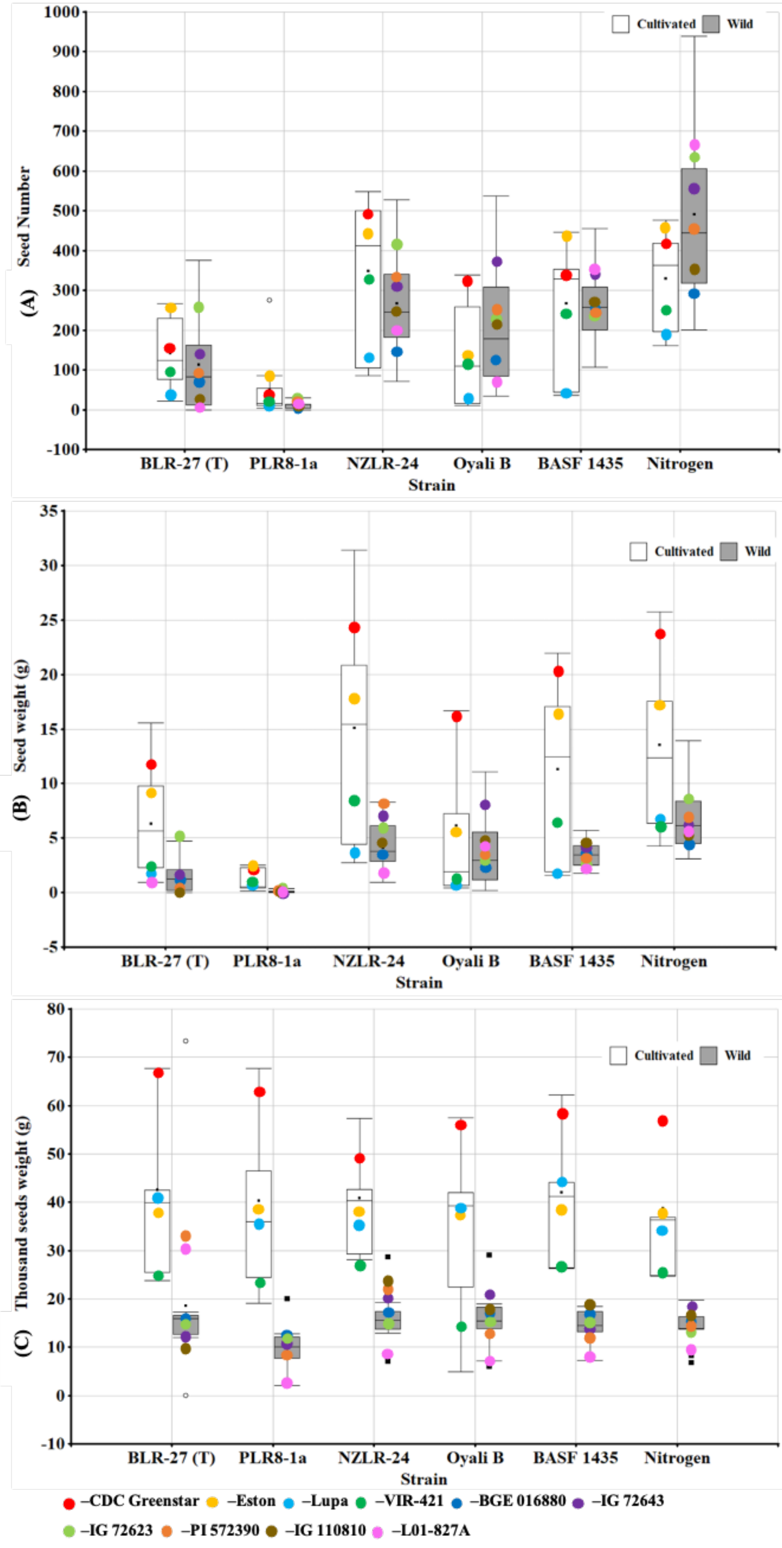

Figure 5.2. A: seed number (LSD 88, P=0.01), B: seed weight (g) (LSD 2.9, P=0.01) and C: thousand seeds weight (g) (LSD 7.3, P=0.01) of 10 *Lens* species accessions inoculated with one of five *Rhizobium* strains or added Nitrogen treatments. Means of cultivated accessions (white box) are displayed separately from the wild ones (grey box).

Seed yield was measured on a per plant basis (SW). Cultivated accessions had mean SW values that were similar to the +N treatment when inoculated with NZLR-24 (*Rlv*) or BASF 1435 (*Rlv*) (Figure 5.2 B). In contrast, most wild accessions had similar mean SW regardless of the strain they were inoculated with. SW means observed in plants inoculated with the strain NZLR-24 (*Rlv*) were the only ones that did not differ from the +N treatment, except for the accessions Lupa and L01-827A. SW in the NZLR-24 (*Rlv*) treatments were higher than those observed in the BASF 1435 (*Rlv*) treatments for the accessions CDC Greenstar, IG 72643 (*L. orientalis*) and PI 572390 (*L. tomentosus*).

The mean SW of IG 72643 (*L. orientalis*) was highest when inoculated with the strain Oyali B (*Rlv*). IG 72623 (*L. odemensis*) had similar SW values when inoculated with BLR-27 (T) (*R. lentis*) compared to the other strains and +N treatment. All interactions with the strain PLR8-1a (*R. bangladeshense*) resulted in poor SW regardless of the accession, and IG 110810 (*L. lamottei*) did not produce any seed when inoculated with this strain.

Overall, there were no significant differences for the mean thousand seed weights (KSW) observed among strains (Figure 5.2 C). CDC Greenstar had a lower KSW when inoculated with the strain NZLR-24 (*Rlv*), compared to +N treatment, as did VIR-421 with the strain Oyali B (*Rlv*). IG 110810 (*L. lamottei*) had the highest KSW when inoculated with NZLR-24 (*Rlv*).

Lupa had higher KSW when inoculated with BASF 1435 (*Rlv*) than the value observed under +N. PI 572390 (*L. tomentosus*) and L01-827A (*L. ervoides*) had the highest KSW when inoculated with the strain BLR-27 (T) (*R. lentis*). KSW for IG 110810 (*L. lamottei*) with the strain PLR8-1a (*R. bangladeshense*) was not defined because no seeds were produced on this treatment.

The highest seed percentage protein was observed for most accessions when inoculated with the strain BASF 1435 (*Rlv*), compared to all other strains and the +N treatment (Figure 5.3 A). Only CDC Greenstar, Eston and Lupa had similar values with +N and higher values with NZLR-24 (*Rlv*) compared to when inoculated with BASF 1435 (*Rlv*)

In general, wild accessions showed higher seed percentage protein values to those observed in the cultivated accessions regardless of the inoculated strain. CDC Greenstar, Eston, Lupa, BGE 016880 (*L. orientalis*) and IG 72643 (*L. orientalis*) had higher percentage protein values when

inoculated with the strain NZLR-24 (*Rlv*) compared to +N. Protein values observed when inoculated with Oyali B (*Rlv*) in Lupa, BGE 016880 (*L. orientalis*), IG 72643 (*L. orientalis*), PI 572390 (*L. tomentosus*) and IG 110810 (*L. lamottei*), were also superior to those observed on the +N treatment. The same higher seed percentage protein values were observed in the accessions BGE 016880 (*L. orientalis*) and IG 72643 (*L. orientalis*) when inoculated with BLR-27 (T) (*R. lentis*) and PLR8-1a (*R. bangladeshense*). Seed percentage protein for BGE 016880 (*L. orientalis*), IG 110810 (*L. lamottei*) and L01-827A (*L. ervoides*) were not determined because no seed was produced, or the sample was not sufficient for conducting the protein analysis.

Protein yield per plant (PY) varied among strains (Figure 5.3 B). Strain mean values for PY were similar between NZLR-24 (*Rlv*) and the +N treatment. Strain mean for PY with Oyali B (*Rlv*) was higher compared to both PLR8-1a (*R. bangladeshense*) and BLR-27 (T) (*R. lentis*). Among the accessions, IG 72623 (*L. odemensis*) had similar PY values when inoculated with either BLR-27 (T) (*R. lentis*) or NZLR-24 (*Rlv*). The accessions CDC Greenstar, IG 72623 (*L. odemensis*) and PI 572390 (*L. tomentosus*) were the only ones that exhibited lower PY values when inoculated with BASF (*Rlv*) compared to NZLR-24 (*Rlv*).

Harvest index (HI) varied most when accessions were inoculated with the strain PLR8-1a (*R. bangladeshense*) and few differences were found with the other strains (Figure 5.3 C). IG 72643 (*L. orientalis*) was the only to have higher HI, compared to the rest of accessions, with the strains NZLR-24 (*Rlv*), BASF 1435 (*Rlv*) and Oyali B (*Rlv*). Eston had significantly lower HI when inoculated with the strain Oyali B (*Rlv*) compared to the +N treatment. VIR-421, IG 72643 (*L. orientalis*) and PI 572390 (*L. tomentosus*) were the only accessions that had similar HI when inoculated with PLR8-1a (*R. bangladeshense*) compared to that observed in the +N treatment. HI of IG 110810 (*L. lamottei*) inoculated with the strain PLR8-1a (*R. bangladeshense*) was zero since there was no seed.

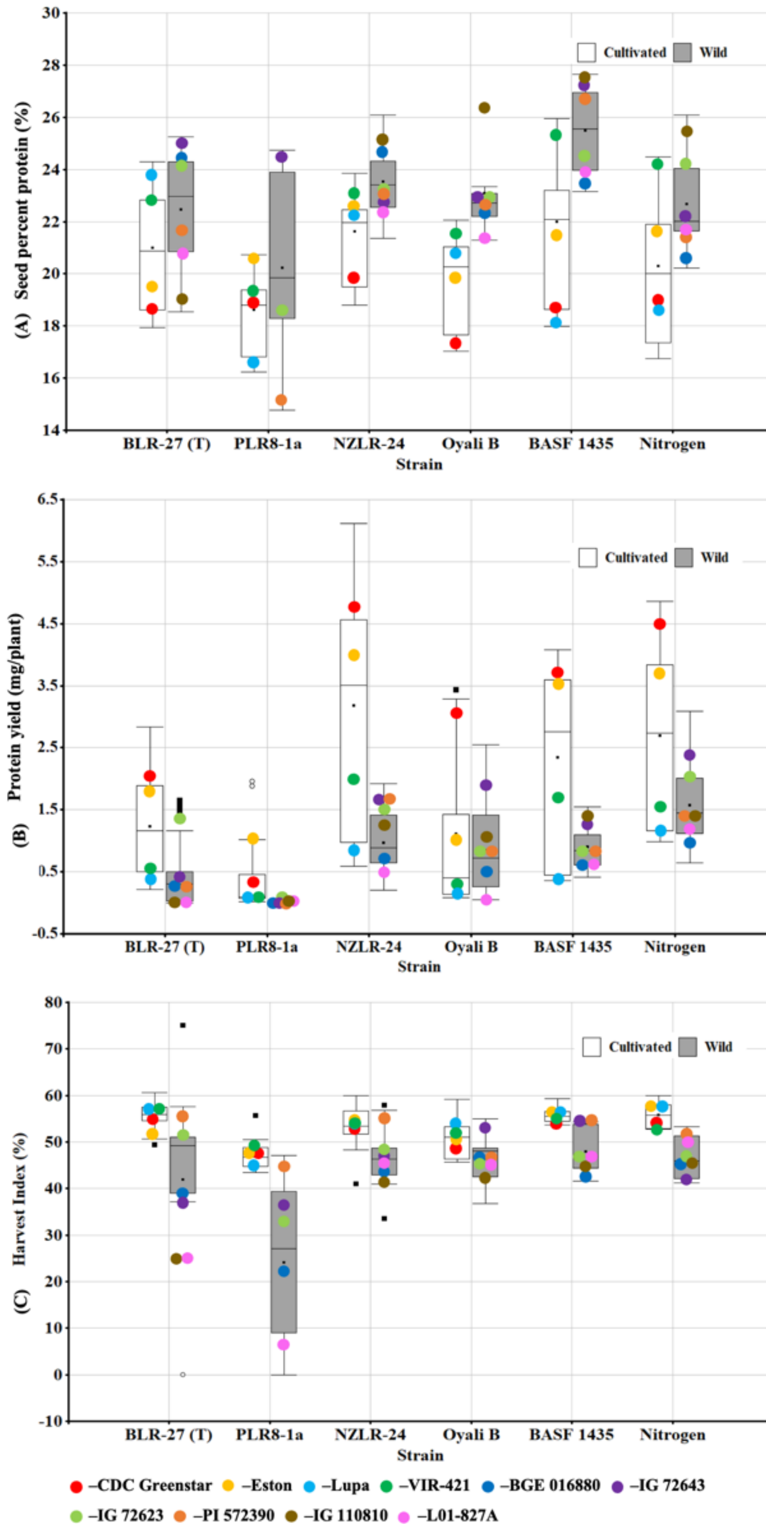

Figure 5.3. A: seed percentage protein (LSD 0.53, P=0.01), B: protein yield (LSD 0.59, P=0.01) and C: harvest index (LSD 0.07, P=0.01) of 10 *Lens* species accessions inoculated with one of five *Rhizobium* strains or added Nitrogen treatments. Means of cultivated accessions (white box) are displayed separately from the wild ones (grey box).

5.3.4. Effect of strains on plant growth.

Accessions changed their biomass in response to strain treatments, and those effects were observed across strains and in some specific cases for accession × strain. All mean values and ANOVAs for section 5.3.4 are in table S.3, Appendix S, and Table O, Appendix O. Different shoot dry weight (SDW) was observed with the inoculation of different strains (Figure 5.4 A). Strain mean of NZLR-24 (*Rlv*) was the highest for SDW among strains. Similar values of SDW were observed between Oyali B (*Rlv*) and BASF 1435 (*Rlv*), while BLR-27 (T) (*R. lentis*) and PLR8-1a (*R. bangladeshense*) exhibited the lowest values. All values of SDW observed with the inoculated strains were lower than those observed on the +N treatment. Among accessions, CDC Greenstar had the highest SDW when inoculated with NZLR-24 (*Rlv*).

Root dry weight (RDW) was also different among all treatments (Figure 5.4 B). The highest strain mean RDW values were observed with the strain BASF 1435 (*Rlv*) followed by the treatment with +N. Intermediate values were observed for those inoculated with NZLR-24 (*Rlv*) and Oyali B (*Rlv*). In contrast to SDW, accessions had higher values of RDW when inoculated with the strain BLR-27 (T) (*R. lentis*) compared to PLR8-1a (*R. bangladeshense*). Differences in RDW were observed for the cultivated accessions, while all wild accessions exhibited similar values under all treatments including +N. CDC Greenstar and Eston had their highest RDW values when inoculated with the strain BASF 1435 (*Rlv*).

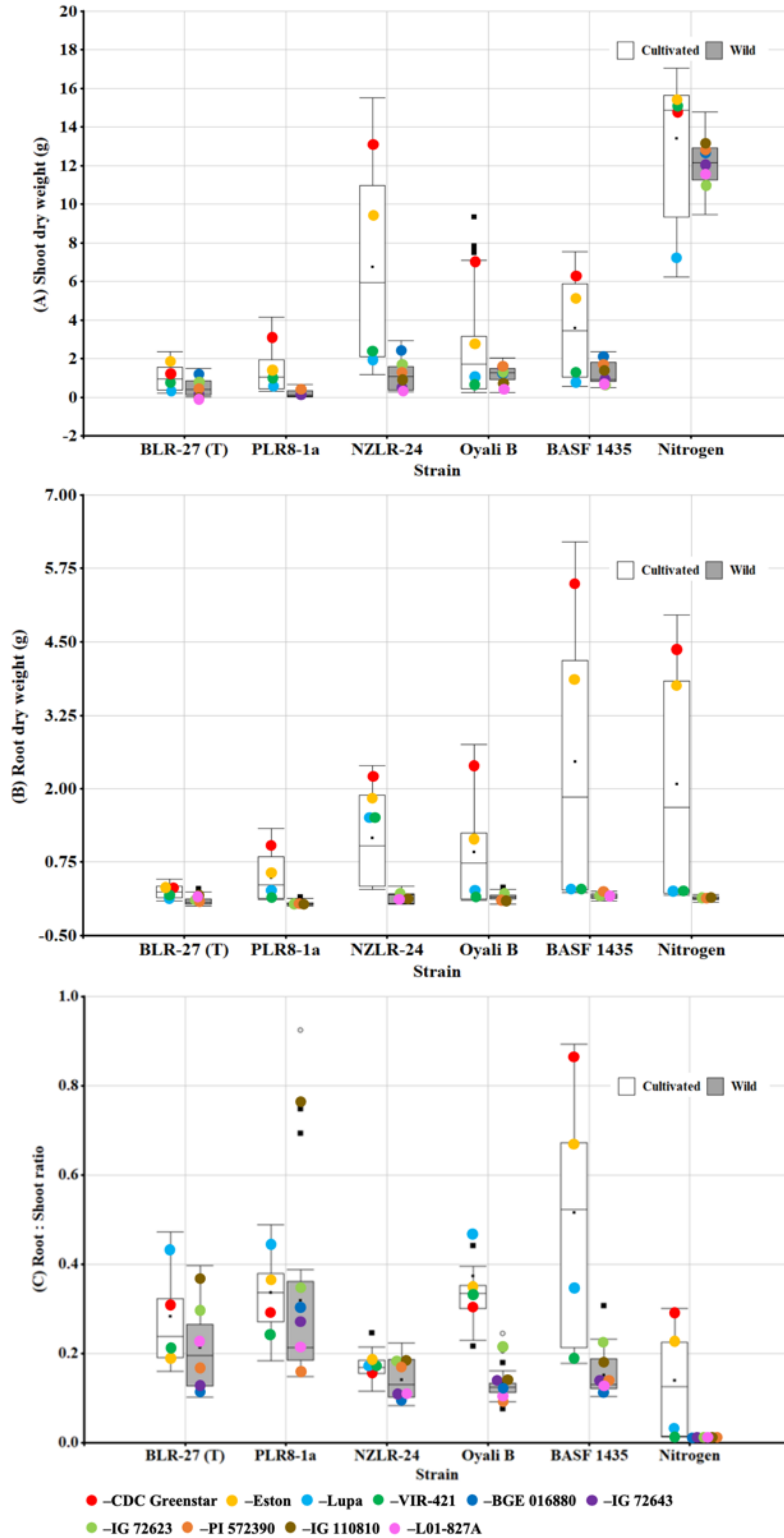

Figure 5. 4. A: shoot dry weight (LSD 0.37, P=0.01), B: root dry weight (LSD 0.1, P=0.01) and C: root to shoot ratio (LSD 0., P=0.) of 10 *Lens* species accessions inoculated with one of five *Rhizobium* strains or added Nitrogen treatments. Means of cultivated accessions (white box) are displayed separately from the wild ones (grey box).

Changes in biomass distribution observed among the strain treatments were reflected on the root to shoot ratios (R:S) (Figure 5.4 C). Accessions exhibited their highest ratios when inoculated with BASF 1435 (*Rlv*) and PLR8-1a (*R. bangladeshense*). Cultivated accessions CDC Greenstar and Eston drastically altered their R:S ratio when inoculated with BASF 1435 (*Rlv*) but the wild accessions did not. With PLR8-1a (*R. bangladeshense*), BGE 016880 (*L. orientalis*) and PI 572390 (*L. tomentosus*) had the highest R:S observed for these accessions among all treatments. Similar R:S values were observed for NZLR-24 (*Rlv*) and +N; the lowest observed among strain means. CDC Greenstar had its lowest R:S when inoculated with the strain NZLR-24 (*Rlv*), and all wild accessions had their lowest values of R:S under the +N treatment.

Strain BLR-27 (T) (*R. lentis*) was the only one that delayed/hastened flowering (DTF) for all accessions relative to +N. The rest of strains did not have any significant impact on DTF (Figure 5.5 A). L01-827A (*L. ervoides*) was the only accession that flowered 10 days earlier when inoculated with BLR-27 (T) (*R. lentis*) compared to +N, opposite to IG 110810 (*L. lamottei*) that had a delay of 13 days with the same strain.

More differences were observed when plants reached days to maturity (DTM) (Figure 5.5 B). No differences were observed among the accessions inoculated with the strains NZLR-24 (*Rlv*) and BASF 1435 (*Rlv*) compared with the +N treatment, but DTM was altered with the other strains. CDC Greenstar matured about a week earlier with PLR8-1a (*R. bangladeshense*), as did Eston with the same strain as well as with Oyali B (*Rlv*). Shorter DTM were also observed in VIR-421 when inoculated with the strains BLR-27 (T) (*R. lentis*), PLR8-1a (*R. bangladeshense*) and Oyali B (*Rlv*). In contrast, Lupa had a delay in DTM when inoculated with NZLR-24 (*Rlv*) relative to the other strains and +N. Such differences in DTM were also observed among the wilds (Figure 5.5 B). IG 72643 (*L. orientalis*) was the only wild genotype to experience a delay relative to +N when inoculated with the strain NZLR-24 (*Rlv*) and also with Oyali B (*Rlv*). IG 72623 (*L. odemensis*) matured earlier with BLR-27 (T) (*R. lentis*), PLR8-1a (*R. bangladeshense*) and Oyali B (*Rlv*). PI 572390 (*L. tomentosus*), IG 110810 (*L. lamottei*) and L01-827A (*L. ervoides*) also matured earlier when inoculated with the strains BLR-27 (T) (*R. lentis*) and PLR8-1a (*R. bangladeshense*). All mean values and ANOVA for DTF and DTM are available in table S. 4 Appendix S, and table O, Appendix O.

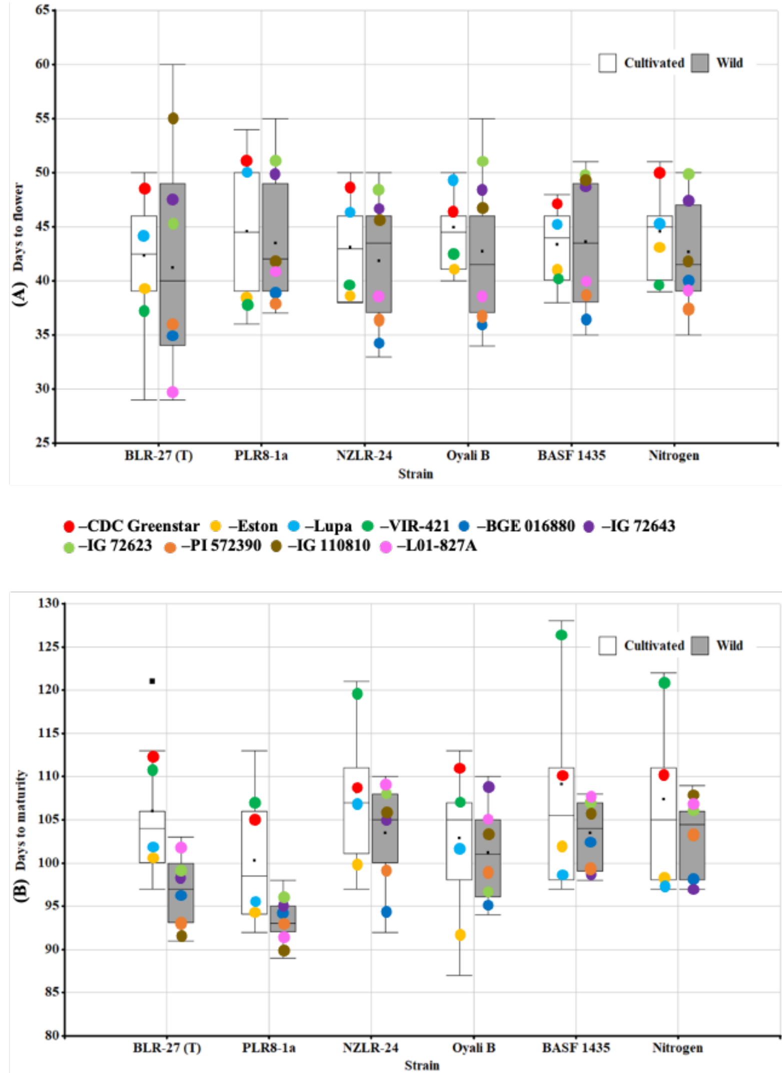

Figure 5.5. A: days to flower (LSD 4.0, P=0.01) and B: days to maturity (LSD 4.09, P=0.01) of 10 *Lens* species accessions inoculated with one of five *Rhizobium* strains or added Nitrogen treatments. Means of cultivated accessions (white box) are displayed separately from the wild ones (grey box).

5.3.5. Correlations among evaluated parameters.

There were strong correlations among N fixed, SDW, SNY, RDW, RNY, SN, SW, PY and efficiency (N fixed/dry nodule weight) (Figure 5.6). N concentration in shoots and roots, R:S and SpecNDW were not correlated with any other parameter measured. Seed percentage protein was not positively correlated to any parameter and had a moderate negative correlation with NN and R:S. NN was also strongly positively correlated with NDW. NFW was correlated with SDW, SNY and N fixed. KSW had a moderate correlation with SDW, RDW, SNY, RNY, N fixed, NN and a very strong correlation with HI.

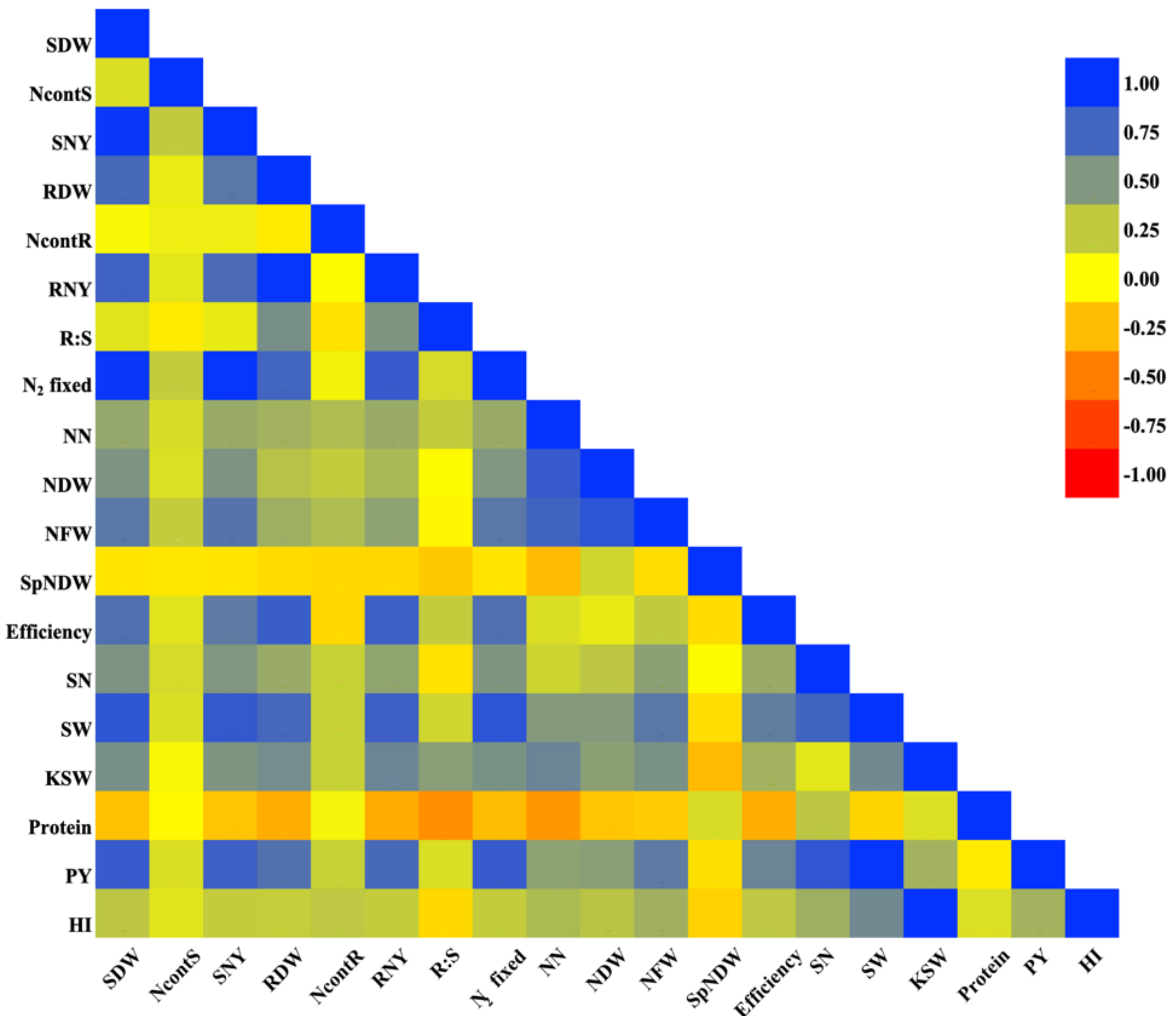

Figure 5.6. Pearson correlation coefficients among parameters evaluated on 10 *Lens* species accessions at 40 DAS and maturity inoculated with one of five *Rhizobium* strains. SDW: shoot dry weight, NcontS: N concentration in shoots, SNY: shoot N yield, RDW: root dry weight, NcontR: N concentration in roots, RNY: root N yield, R:S: root to shoot ratio, N_2 fixed, NN: number of nodules, NDW: nodule dry weight NDW, NFW: nodule fresh weight, SpNDW: specific nodule dry weight, Efficiency: efficiency of nodulation, SN: seed number, SW: seed weight SW, KSW: thousand seeds weight, Protein: seed percentage protein, PY: protein yield and HI: harvest index.

5.4. DISCUSSION

The objective of this study was to characterize N fixation related traits among specific combinations of a selected group of *Lens* and *Rhizobium* species to determine the level of the accession × strain specificity and the effectiveness of this relationship. This included effects on parameters at flowering, phenotypic characterization of the nodules, as well as effects on seed

production and N-related seed quality traits. This is important for breeding processes given that the interaction of the two components has to be established to determine the most suitable genetic resources of both components before starting to breed for enhancing this ability.

Preliminary desirable characteristics within both the cultivated and wild *Lens* species were reported in Chapter 3 that called for further validation with a greater set of strains. In addition, the best strain partners under local field conditions were reported in Chapter 4. Here, the range of the superior N fixing ability of both symbiotic partners was established for multiple species of both *Lens* and *Rhizobium*.

Evaluating combinations of such diversity validated the differential ability to enter into the symbiotic relationship that exists between wild and cultivated *Lens* and how domestication has impacted the effectiveness of this process. Seed production in wilds was, in general, more efficient than in the cultivated accessions when inoculated with a range of *Rhizobium* strains, based on comparisons to the +N treatment with optimum N fertilization. Differential seed protein concentration was observed between both groups with exceptionally high values observed only among the inoculated wild accessions. The clear difference in nodule type - indeterminate in most wild vs determinate in cultivated, is quite likely contributing to the increased efficiency in the wilds.

Results also provided a better understanding of the role of diverse strains in the symbiotic relationship and how both components interacted. Strains demonstrated a differential ability for N fixation related traits as well, with a range of efficiency across diverse *Lens* germplasm. Those that demonstrated a broader effective fixing capacity, such as NZLR-24 (*Rlv*) are proposed for selection processes in breeding for better BNF ability in the future.

Effects at the accession × strain level have been reported previously within a group of five lentil varieties from the USDA inoculated with 15 *Rlv* strains (Abi-Ghanem *et al*., 2011). In this group, differential effects of cultivar, strain and interactions between accession × strain were observed for their N fixing ability. This earlier work suggested the importance of breeding for better BNF in lentils, the necessity to do such breeding selection with top performing strains, and the need to determine the range of N fixation ability of the studied materials with multiple interactions.

Seed weight (SW) was clearly affected by the strain used to inoculate the different accessions. The inoculations with NZLR-24 (*Rlv*) resulted in SW similar to that observed under +N in both cultivated and wild *Lens* accessions. This strain performed the best in a multi-site-year field trial in Saskatchewan that tested a diversity of lentil-nodulating *Rhizobium* species with one lentil cultivar and showing its stability across environments (Chapter 4). Furthermore, it is ideal to select strains that can also infect other economically important species in the area, and NZLR-24 (*Rlv*) can infect pea and faba beans (*Vicia faba*) (Chapter 4). The ability to efficiently infect a diverse set of *Lens* germplasm found in this study, and its stability under target agro-environmental conditions, showed its value for improving inoculants as well as for selection processes for better BNF ability in the breeding program.

In addition to NZLR-24 (*Rlv*), inoculations with Oyali B (*Rlv*) produced similar yield values to those observed with the commercial strain BASF 1435 (*Rlv*). This also represent an interesting resource because it is a strain isolated from Turkish soil, around the centre of origin of lentils. *Rhizobium* from wild legumes has been recognized as an important focus of study in lentils, because of their diversity, promiscuity and ability to survive under arid conditions, as well as their ability to colonize wild and domesticated plants equally (Zahran 2001). PLR8-1a (*R. bangladeshense*) and BLR-27 (T) (*R. lentis*), from Pakistan and Bangladesh respectively, were selected as representatives of other species that nodulate lentil and performed similarly to the commercial strain BASF 1435 (*Rlv*) in in the multi-site-year field trial (Chapter 4), but neither exhibited generally desirable N fixing characteristics with this more diverse group of *Lens* species.

Accessions also played a differential role on the effectiveness of this relationship as measured by SW. CDC Greenstar, Eston and VIR-421 had SW results with one or two strains that were not different from +N. With the exception of L01-827A (*L. ervoides*), the rest of the wilds had yields that were similar to the +N with 2 or more strains. IG 72643 (*L. orientalis*) had similar SW to those observed with +N with 3 strains: NZLR-24 (*Rlv*), Oyali B (*Rlv*), BASF 1435 (*Rlv*). This ability to efficiently establish relationships with a greater range of symbionts was not observed in any cultivar tested. Promiscuity (the ability to efficiently establish relationships with a greater number of symbionts) is important because it gives accessions a greater opportunity to function in the presence of more diverse symbionts, but it was not observed in any cultivar.

Some combinations exhibited a high level of specificity. IG 72623 (*L. odemensis*), from Turkey, was the only accession to have a favorable yield response with the strain BLR-27 (T) (*R. lentis*). PI 572390 (*L. tomentosus*), also from Turkey, did not have a favorable yield response with this strain, however. IG 72623 (*L. odemensis*) also had a favorable yield response with other strains, however, which suggest that the high level of specificity is conditioned by the strain BLR-27 (T) (*R. lentis*) or the species *L. odemensis*, and it does not necessarily have any relation to the geographic origin of these accessions.

Wild accessions could make contributions to breeding for higher seed percentage protein *per se*, but findings from this experiment suggest that optimizing the symbiotic ability in *Lens* could have a greater impact. Both seed percentage protein and protein yield (PY) were measured because they were not expected to be correlated and are both important. As expected, seed percentage protein was not correlated to any other measured parameter, but PY was affected by SDW, SNY, RDW, RNY, NFW and N fixed, which is directly related to SW as shown by the correlation coefficient of 0.99 between SW and PY. Most wild accessions not only had higher values of seed percentage protein compared to cultivated accessions but were also higher compared to their respective fertilized controls.

Among the strains, BASF 1435 (*Rlv*) had 1- 4.5% higher values of seed percentage protein when compared to +N and all the other strains. Among accessions, IG 72643 (*L. orientalis*) had values of seed percentage protein values from 22.1-27.2 %; higher than that observed in any of the cultivated accessions. These superior values are observed across strains but exhibit greater differences at the accession × strain level. IG 72643 (*L. orientalis*) that had an average value of seed percentage protein of 24.0%, had a mean of 27.2% with BASF 1435 (*Rlv*), likewise in the interaction between IG 110810 (*L. lamottei*) × BASF 1435 (*Rlv*), 27.1% protein was observed, compared to the average value of 23.6% across all treatments.

The presence of indeterminate nodules, in greater proportions and bigger phenotypic sizes (higher specific nodule dry weight-SpNDW) only in the wilds was the main difference observed in the nodulation system between cultivated and wild types, therefore, this could play a role in the ability of the wilds to reach higher SW and protein content compared to most cultivars. Indeterminate nodules fix N longer compared to the determinate nodules observed in the cultivars. A good example of this was the particularly higher proportion of indeterminate nodules

observed in IG 72623 (*L. odemensis*) when inoculated with the strain with BLR-27 (T) (*R. lentis*) (Figure R, Appendix R) which resulted in higher SW values for this specific relationship. This phenomenon is worth further exploration to determine if it can be transferred to cultivated lentil.

The SpNDW differences found among *Lens* accessions, with no effects of strain, suggested it is control by the plant genotype alone. The lower values of SpNDW observed in some specific wild accessions × strain interactions, can be explained by the distribution of different nodule phenotypes in the same plant (Figure R, Appendix R), particularly in those with poor nodulation and symbiotic characteristics. A good example of this is the phenotypic distribution observed on the wild accession BGE 016880 (*L. orientalis*) when inoculated with the strain PLR8-1a (*R. bangladeshense*), in which half of its nodules were indeterminate palmate of bigger size, but the other half were very small determinate types. Those smaller nodules were observed to be developed in the latest formed roots, probably just days before plants were sampled and could also be ineffective. The specificity of nodule phenotypes observed at the accession × strain level, has been observed in medicago, where the difference between determinate and indeterminate nodule types is attributed to the *MtNFH1* Nod Factor (NF) (Cai *et al*., 2018). An approach for better understanding this characteristic in lentils could be studying the segregation of this characteristic in interspecific population with *Lens* parents with different nodule types.

It was not unexpected that the majority of accessions responded with significant biomass increases under optimum fertilization as compared to inoculation, as it is generally an easier way to obtain N (Postgate 1982, Chapter 3). Also, most of the N was allocated in shoots and only a small portion in roots (Figure P, Appendix P). These results are consistent with observations made under similar experimental conditions in peas (Yang *et al*., 2017). The root to shoot ratio was mainly affected by interactions with the strain BASF 1435 (*Rlv*). Higher root to shoot ratios were also observed with this strain under greenhouse conditions for some specific accessions when compared to +N (Chapter 3). It is known that efficient N fixation makes allocation of root biomass independent of soil N supply (Markham and Zekveld 2007). The higher proportion of roots under this strain, suggests its lower efficiency compared to compared to +N and the strain NZLR-24 (*Rlv*). Higher root allocation might also have a role in its lower yield performance.

The effectiveness of the accessions × strain interaction is determined by its yielding capacity (SW) (Unkovich *et al*., 2008). Shoot parameters SDW and SNY, as well as N fixed, were the

best parameters measured at flowering for predicting SW and PY. SDW had a high correlation to SW because no mineral soil N was available, therefore it is a reliable measure. This would not be the case in the presence of any available mineral soil N due to the differential ability legumes, including lentil, have in using those resources (Azam and Farooq 2003, Chapter 3). As expected, the nodule efficiency rate (N fixed/dry nodule weight) was highly correlated with N fixed, however was not the best predictor for SW.

Measures of nodulation such as NDW and NN have been used extensively for measuring BNF ability and to discriminate among strains and cultivars (Koskey *et al*., 2017, Munoz *et al*., 2016, Hafeez *et al*., 2000, Abi-Ghanem *et al*., 2011, Ruiz-Diez *et al*., 2012a). As demonstrated in peas (Chao *et al*., 2017) and observed in this experiment, however, nodule activity seems to be more important than nodule production when determining the effectiveness of this relationship. Neither NDW nor NN had a strong positive correlation with any other parameter measured. It is also possible that the diversity in nodule phenotypes and broad range of sizes makes the estimation of NDW inaccurate because of the differences in surface area. In addition to this, ineffective nodulation (formation of nodules that do not fix N) may be contributing to the lack of correlations. Ineffective nodulation was the case in the combination IG110810 (*L. lamottei*) × BL-27(T) (*R. lentis*), that had in total 25 nodules, none of which fixed any N. NDW was used as it is the standard measure for dry biomass and the required parameter for estimating efficiency. However, fresh weight of nodules (NFW) was better correlated not only to SNY, but also to SDW, N fixed and SW, which suggests it is a more suitable measure when evaluating genotypes with diverse phenotypic nodulation systems.

Days to flower were only affected in treatments with the strain BLR-27 (T) (*R. lentis*). Days to maturity was affected only for specific interactions, mainly with BLR-27 (T) (*R. lentis*) and PLR8-1a (*R. bangladeshense*) that were generally the strains with the most undesirable N fixing characteristics. Conducting this experiment under controlled conditions gives the general advantage of estimating N fixed accurately with a relatively simple method. However, field experimentation will be required for a better understanding of how this symbiosis is affecting days to flower and days to maturity and in general for better understanding environmental effects on this relationship, especially for validating accessions of interest for breeding. Selection should be based ideally on a combination of methods, including precise data generated under controlled

conditions that provides accurate information on genotype's ability to fix N, and field-based data, that will potentially reduce heritability values, but will consider the environmental effects on the efficiency of this relationship.

The results obtained in this study demonstrate the ability of the strain NZLR-24 (*Rlv*) to establish efficient relationships with individuals from 5 *Lens* species: *Lens culinaris* cultivars and accessions from 4 wild species. It is also clear that wild *Lens* species are more promiscuous than cultivars, have more stable yields when inoculated, and can produce seeds with exceptionally high protein content when inoculated with various *Rhizobium* strains. Wild accessions BGE 016880 (*L. orientalis*), IG 72623 (*L. odemensis*) and IG 72643 (*L. orientalis*) represent good sources of positive variability. Evaluating the inheritance of their abilities inoculated with the strain NZLR-24 (*Rlv*) will more effectively establish their potential contributions to the BNF ability in the lentil crop.

Prologue to Chapter 6

Specific genotypes of *Rhizobium* and *Lens* were identified for their promiscuous ability, and more efficient N fixing ability and reported in Chapter 5. This represents promising germplasm for the improvement of the BNF ability in lentils. Differential phenotypic nodulation structures were identified among all wild genotypes that differentiate them from cultivated *Lens* (Chapter 5). How these characteristics are inherited are reported in Chapter 6.

The data obtained in Chapter 6 were delayed by, and then measured under, COVID-19 restrictions, therefore, phenotyping and subsequent analyses were limited by the schedule and conditions established for the use of phytotron and laboratory facilities. Further analyses will be carried out once access to the labs is easier and a full manuscript will be developed at that time.

Paper and co-author contributions will be as follow:
Vargas, A. and K.E. Bett. 2021. Genetic characterization of BNF-related traits in three interspecific lentil populations.

Author contributions:
Vargas, A.: responsible for designing concepts, execution, analysis of experiments and preparation of manuscript with input from Bett, K.E.
Bett, K.E.: funding acquisition, supervised Vargas, A., revising and editing the manuscript.

6. GENETIC CHARACTERIZATION OF BNF-RELATED TRAITS IN THREE INTERSPECIFIC LENTIL POPULATIONS

6.1. INTRODUCTION

There has been extensive research done measuring the impact of the symbiotic relationships between legumes and rhizobia, the agro-environmental effects on this relationship, and its contributions to cropping systems (Howieson and Dilworth 2016; Unkovich *et al*., 2008, Herridge *et al*., 2008; Unkovich and Pate 2000). As has already been pointed out in several reviews, however, there has only been limited research towards breeding legume crops for increased biological nitrogen fixation (Coba de la Pena and Cueyo 2012; Graham *et al*., 2004; Knight 2003).

It has been proposed that the ability to fix N has been negatively impacted by selection under high fertility conditions (Wissuwa *et al*., 2009), which, as demonstrated in *Lotus japonicus*, is suppressed in the presence of nitrate fertilizer (Nishida *et al*., 2021). Also, genotypic differences observed in the nodulation signalling pathways among individuals of different legume species suggests the importance of selecting germplasm with higher ability to efficiently interact with rhizobia (Rengel 2002). Introgressiomics, an approach for trait introgression from wild relatives using phenotypic and genomic data (Prohens *et al*., 2017) is used by many breeding programs for regaining the diversity crops need to meet the current agriculture goals (Maxted and Kell 2009).

For instance, in common bean (*Phaseolus vulgaris*), the symbiotic phenotypic characteristics of some interspecific hybrids with its wild relative tepary bean (*Phaseolus acutifolius*) is superior to that of the domesticated parent (Somasegaran and Hoben 1994). On the other hand, in soybean (*Glycine max*), it has been proposed that improved N fixing ability was selected indirectly when selecting for bigger plants and larger seeds and a differentiated, more adequate carbon/nitrogen (C/N) balance compared to the wild type (*Glycine soja*), giving domesticated soybean a higher symbiotic ability (Munoz *et al*., 2016).

Assessing BNF studies requires multiple phenotypic measurements, often under various systems simultaneously. Given the labor-intensive phenotyping required to measure these type of traits, indirect selection of linked markers would be ideal for a breeding program (Wissuwa *et al*., 2005). The first QTL responsible for N fixation traits were reported in the model legume *Lotus*

japonicus for, among other traits, nodule number and nodule weight (Tominaga *et al.,* 2012). QTL have been linked to nodule number and size in soybean (Yang *et al*., 2019), while in common bean they have been associated with N fixation traits including N derived from the atmosphere (Ndfa) (Kamfwa *et al*., 2019, Heilig, *et al*., 2017) and in cowpea (*Vigna unguiculata*) with inefficient nodulation and N utilization related traits (Ohlson *et al*., 2018; Seido *et al*., 2019). The only QTL reported for this ability in an interspecific population were in soybean - for nodule fresh weight and total ureides (Munoz *et a*l., 2016). Markers related to nodulation traits in lentil would be of utility, especially when crosses with wild parents are being made.

There are wild accessions among the genus *Lens* with higher nitrogen fixation ability than the domesticated species (Chapter 3), *Rhizobium* strains that represent potential new resources with higher performance under local conditions compared to commercially available products (Chapter 4), and promiscuous lentil genotypes that exhibited a high range of efficiency in multiple combinations (Chapter 5). Recombinant inbred line (RIL) populations LR-68 and LR-70 were developed at the Crop Development Centre (CDC) of the University of Saskatchewan to introduce disease resistance into modern cultivars. RIL population LR-86 was developed at the University of Leon in Spain (Fratini *et al*. 2007). The parental lines of these three populations have differential nodulation characteristics and N fixing ability (Chapters 3 and 5), making them ideal for assessing the inheritance of these traits. They have also already been genotyped, facilitating quantitative trait loci (QTL) mapping of nodulation traits.

The first objective of this study was to conduct a phenotypic characterization of the interspecific populations LR-68, LR-70 and LR-86 for their N fixation related traits when inoculated with the strain NZLR24 (*Rlv*). The second, identify QTL responsible for nodulation traits that will provide breeders with breeding tools for increasing N fixation ability.

6.2. MATERIALS AND METHODS

6.2.1. Interspecific populations and *Rhizobium* strain

The LR-68 (IG 72643 *L. orientalis* × CDC Greenstar *L. culinaris*), LR-70 (Eston *L. culinaris* × IG 72623 *L. odemensis*) and LR-86 (Lupa *L. culinaris* × BGE 016880 *L. orientalis*) recombinant inbred line (RIL) populations (list of RILs in Tables T.1, T.2, T.3, Appendix T) were evaluated for their symbiotic ability. Selection of these populations was based on the differential ability to

nodulate, fix N and produce seeds of their parental lines (Chapters 3 and 5). The LR-68 parental lines were among the highest N fixers, had high seed yield with *Rhizobium* and exhibited desirable N fixation related traits in general. For the LR-70, Eston had intermediate performance and IG 72623 (*L. odemensis*) had high N fixation ability among the wilds. The LR-86 population has the cultivar parent Lupa with poor symbiotic ability and its wild parent, BGE 016880 (*L. orientalis*), had intermediate symbiotic performance among the wild parental accessions. The three populations were inoculated with the *Rhizobium* strain NZLR-24 (*Rlv)* that was selected after performing the highest under local field conditions (Chapter 4) and its ability to infect and efficiently nodulate diverse *Lens* species germplasm (Chapter 5).

6.2.2. Growing conditions and inoculum

The experiments were conducted in a growth chamber in the phytotron facility at the Agriculture Building, U of S during 2020. The chamber was set to a 16-hour day length and temperature was set to 21 ˚C during the day and 15 ˚C at night. Twenty seeds of each line were disinfected, scarified and pre-germinated in soft agar (6% w/v) for 24-48 hours. For disinfection, seeds were surface sterilized with 70% ethanol (v/v) for 30 seconds, followed by 5% bleach (v/v) for 2 minutes, and washed with running distilled water. Scarification was conducted to ensure germination of the wild accessions and carried out manually by nicking the seed coat with a razor blade before plating.

Inoculum of the strain NZLR-24 (*Rlv*) was grown in yeast mannitol broth (YMB, Sigma Aldrich #Y3377), agitated in a shaker (Orbital, Thermo Scientific, Waltham MA) for 48 hours at 26 ˚C and 180 rpm and standardized to 3×10^9 cells/ml. Three (LR-68 and LR-70) or four (LR-86) germinated seeds with similar primary root length (0.5 - 1.0 cm) were transfer to plastic growing pouches in black boxes with nutrient solution. Boxes had previously been disinfected with 70% ethanol and all other equipment was autoclaved during 1 hour at 121℃ and 15 psi to avoid growth of molds during the experiment. The growing pouches consisted of blue blotter paper (215.9 × 279.4 × 31 mm) (Anchor Ltd., St. Paul MN, USA) covered with plastic sheet protectors of the same size. Pouches were hung from wooden sticks (32 cm long) in black plastic file boxes (46.1 × 35.9 × 27.3 cm) (Figure 6.1 A). Seedlings were transferred with forceps to avoid damage of the primary root. Once transferred, seedlings were inoculated with the liquid culture by applying 1 ml of liquid inoculum directly to the germinated seedling (Figure 6.2 B). Thirty-

eight pouches were placed in each box. N- free nutrient solution (Broughton and Dilworth, 1970, Table M, Appendix M) was added to cover the bottom 10% of the pouch to allow the nutrient solution to slowly move to the area where the seedlings were placed. Boxes were covered with aluminum foil leaving a 2 cm space in the center part for plants to grow (Figure 6.1 C). Nutrient solution was added every four days as needed by the plants.

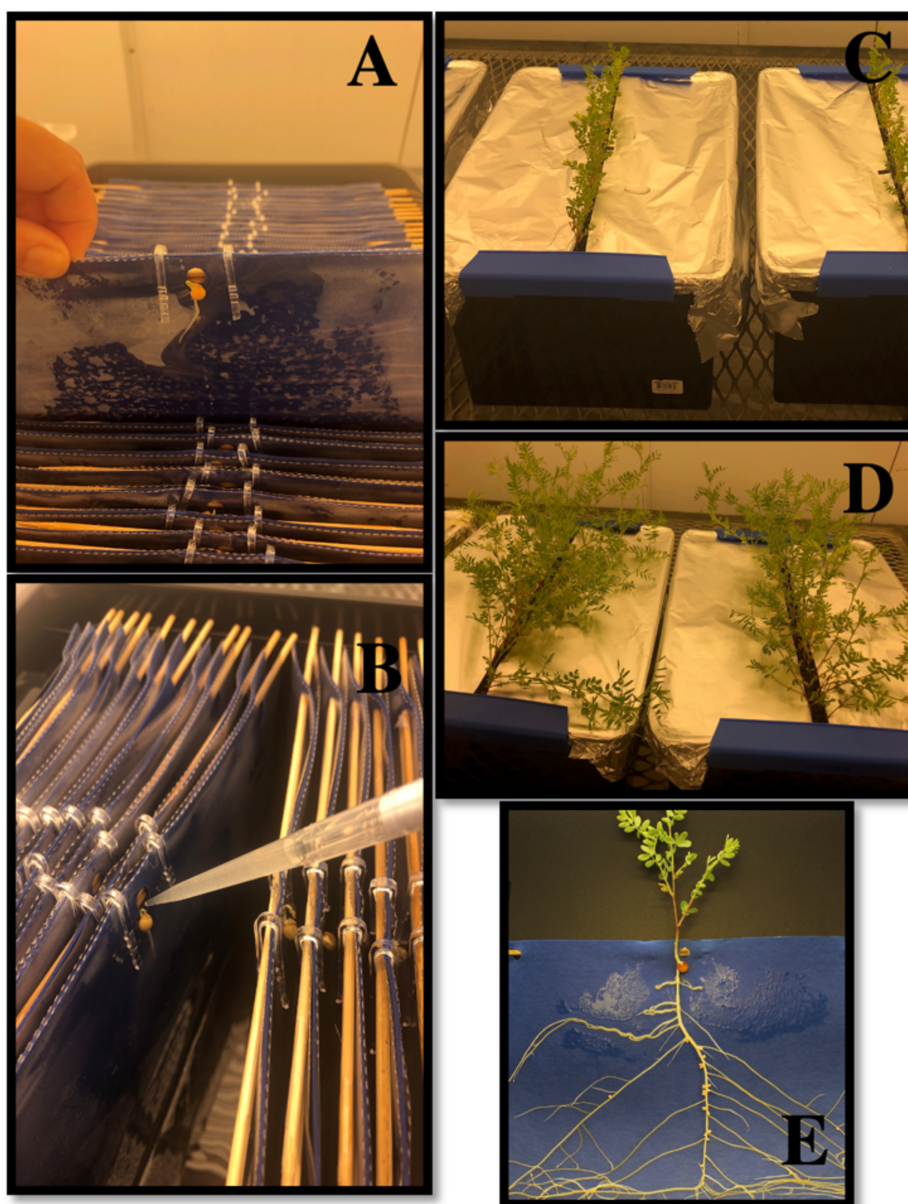

Figure 6.1. A: germinated seed transferred, B: inoculation, C: plants established in black boxes, D: plants at 30 DAS and E: pouches at 30 DAS. System utilized to evaluate the interspecific populations LR-68, LR-70 and LR-86 inoculated with the strain NZLR-24 (*Rhizobium leguminosarum* bv. *viciae*), grown in a cabinet in plastic pouches and evaluated for N fixation related traits 30 DAS.

6.2.3. Phenotypic parameters

All parameters were measured 30-32 days after seeding (DAS) (Figure 6.1 D, E). A full replicate was phenotyped per day. Shoots were separated from roots with scissors, and nodules were separated manually from the root. Shoot fresh weight (SFW) and root fresh weight (RFW) were recorded. Because units were extracted from their growing pouch at the moment of phenotyping, fresh weights were considered accurate enough. Nodules were counted (NN) and fresh weight (NFW) of all nodules from a plant were recorded for each plant. Shoots and roots were dried at 70 ˚C for 72 hours to determine shoot and root dry weight (SDW, RDW). Nodules were dried at 60 ˚C for 48 hours to determine nodule dry weight (NDW). Specific nodule weight (SpNFW, SpNDW) was estimated by dividing NFW and NDW by NN.

6.2.4. Experimental design and statistical analysis

One population was planted at a time due to space constraints. Parental lines were included in each experimental run for reference. Three repetitions of each line were established of the 121 and 119 individuals of the populations LR-68 and LR-70, respectively, and 4 repetitions of the 93 individuals of the LR-86 population, for a total of 1112 experimental units. For the statistical analysis of the phenotypic characteristics, lines were considered as a fixed effect and replications as a random effect. Broad sense heritability was calculated as $H^2= \sigma^2G/ (\sigma^2G+ \sigma^2e/b)$, where σ^2G was the genotypic variance, σ^2e the residual variance, and b was the number of replications. Phenotypic Pearson correlation coefficients were estimated among all measured parameters. Testing of the data for normal distribution was done using the Shapiro-Wilk test, and homogeneity of variance was tested with a Levene's test. The ANOVA, variance components and the phenotypic Pearson correlation coefficients were estimated using the 'variability' function in R (Popat *et al*., 2020). The phenotypic data met the assumptions of an ANOVA.

6.2.5. QTL detection analysis

These populations were previously genotyped and genetic linkage maps were constructed by the lentil genetics program at the University of Saskatchewan. Files with the genotypic data and linkage groups (LG) are available for download from (https://knowpulse.usask.ca/search/genetic-maps). For the LR-68 population, 118 individuals were included in the QTL association analysis with 614 single nucleotide polymorphism (SNP) markers. For the LR-70 population, 111 individuals with 651 SNP markers were used, and for the LR-86 population, 87 individuals with

4073 SNP markers were used. The individuals phenotyped and included in the QTL analysis are indicated in Tables T.1, T.2, T.3, Appendix T. LS-means of replicates were used for the QTL mapping of each population separately. The analysis was performed using inclusive composite interval mapping (ICIM) using IciMapping v4.2 (Meng *et al*., 2015). Analysis of the additive effects at individual QTL were determined with a critical logarithm-of-odds (LOD) based on 1000 permutations and 1 cM walking step at a 0.05. Initial QTL detected for the phenotypic traits NFW, NDW, SpNFW, SpNDW and NN, were integrated based on the position of their confidence interval to construct the QTL and displayed in the linkage map of each population as reported in previous studies (Yang *et al*., 2017). To investigate whether the same or different QTL were mapped in the three populations, linkage map positions were aligned with known physical positions for markers on the cultivated lentil genome v2.0 (Ramsay *et al*., 2019) to estimate physical positions for QTL and presented separately as Meta-QTL.

6.3. RESULTS

6.3.1. Variability and distribution of N fixation related traits

The estimates of the variance components and H^2, ANOVA and means for results for all traits studied in the three populations are in Tables U, V, W, Appendices U, V, W. There were highly significant differences among lines for all traits measured in all three populations. In the distribution of the LR-68 and LR-70 populations, the parents had contrasting values for all fresh and dry weights and transgressive segregation was not evident (Figure 6.2). Transgressive segregation was observed, however, in the population LR-86 in the direction of higher values compared to both parents for all fresh and dry weight measurements (Figure 6.2 A, B, D, E, G, H).

The RILs of the LR-86 population had a wider range of variation for SFW, SDW, RFW, NDW, SpNFW and SpNDW. All three populations had similar variation on their RDW (Figure 6.2 E). The greatest variation for the NN was observed among the RILs of the LR-70 population (Figure 6.2 I). RILs from the LR-86 population exceeded all SFW, SDW, RFW and RDW values observed compared to the other two populations. For NFW and NDW (Figure 6.2 G, H) the RILs from all populations skewed towards lower nodule weight values.

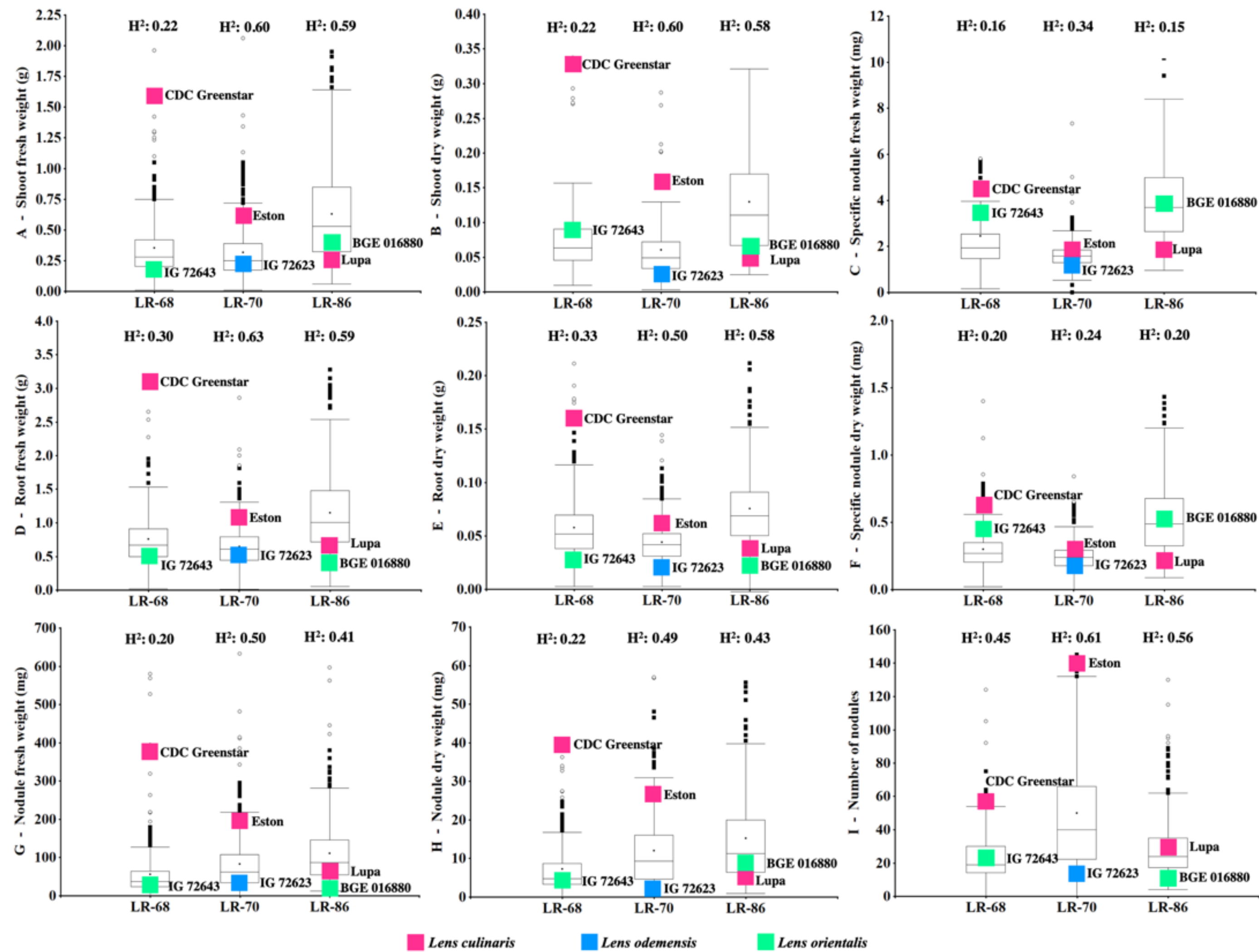

Figure 6.2. Distribution of A: shoot fresh weight (SFW), B: shoot dry weight (SDW), C: specific nodule fresh weight (SpNFW), D: root fresh weight (RFW). E: root dry weight (RDW), F: specific nodule dry weight (SpNDW), G: nodule fresh weight (NFW), nodule dry weight (NDW) and I: number of nodules (NN) in RIL of the LR-68 (IG 72643 ×CDC Greenstar), LR-70 (Eston × IG 72623) and LR-86 (Lupa × BGE 016880) interspecific populations inoculated with the strain NZLR-24 (*Rhizobium leguminosarum* bv. *viciae*), grown in a cabinet in plastic pouches and evaluated 30 DAS. H^2: broad sense heritability.

The specific nodule weight values were distributed differently compared to all weight values (Figure 6.2 C, F). The LR-68 population exhibited transgressive segregation for both SpNFW and SpNDW towards values lower than the parents which had similar values. In contrast, in the LR-86 population parents showed contrasting values, and distribution of the RILs was skewed towards lower SpNFW and SpNDW (Figure 6.2 C, F). For NN, parental lines showed contrasting values in the distribution. All *L. culinaris* parents had higher numbers of nodules compared to the respective wild parent in all three populations (Figure 6.2 I). All three RIL populations were skewed towards lower NN. The highest NN were observed among the individuals of the LR-70 population.

Low estimates of broad sense of heritability (H^2) were obtained for most traits evaluated in the LR-68 population, except for NN which was moderate (Figure 6.1 I). Moderate to high estimates were observed for the LR-70 and LR-86 RILs for most traits, except for SpNFW and SpNDW which were low. The highest H^2 for NN was observed in the LR-70 population.

6.3.2. Phenotypic correlations

The strongest correlation coefficients among N fixation related traits were observed among SFW, RFW NFW, SDW, RDW and NDW for all three populations (Figure 6.3). In the LR-68 the correlation between NFW and the other parameters was not as strong as those observed in the other two populations. The correlation coefficient observed between RDW and NDW in the LR-70 population was lower compared to the other two populations.

SpNFW and SpNDW were not strongly correlated to any of the root or shoot weights. NN had a moderate correlation to most parameters among the individuals of the LR-68 population, only strongly correlated to NDW, and not correlated to SpNFW or SpNDW. Similar correlations were observed between NN and the plant weight parameters among the LR-86 RILs, with higher correlations between NN and NFW than those observed among the LR-68 RILs. The correlations between NN and the other parameters among the LR-70 RILs were the strongest, compared to the other populations, except to RDW. SpNDW had a strong correlation only with NFW in the LR-68 and a moderate correlation to NDW among the individuals of the LR-68 and LR-86. All correlation coefficients are available in Table X, Appendix X.

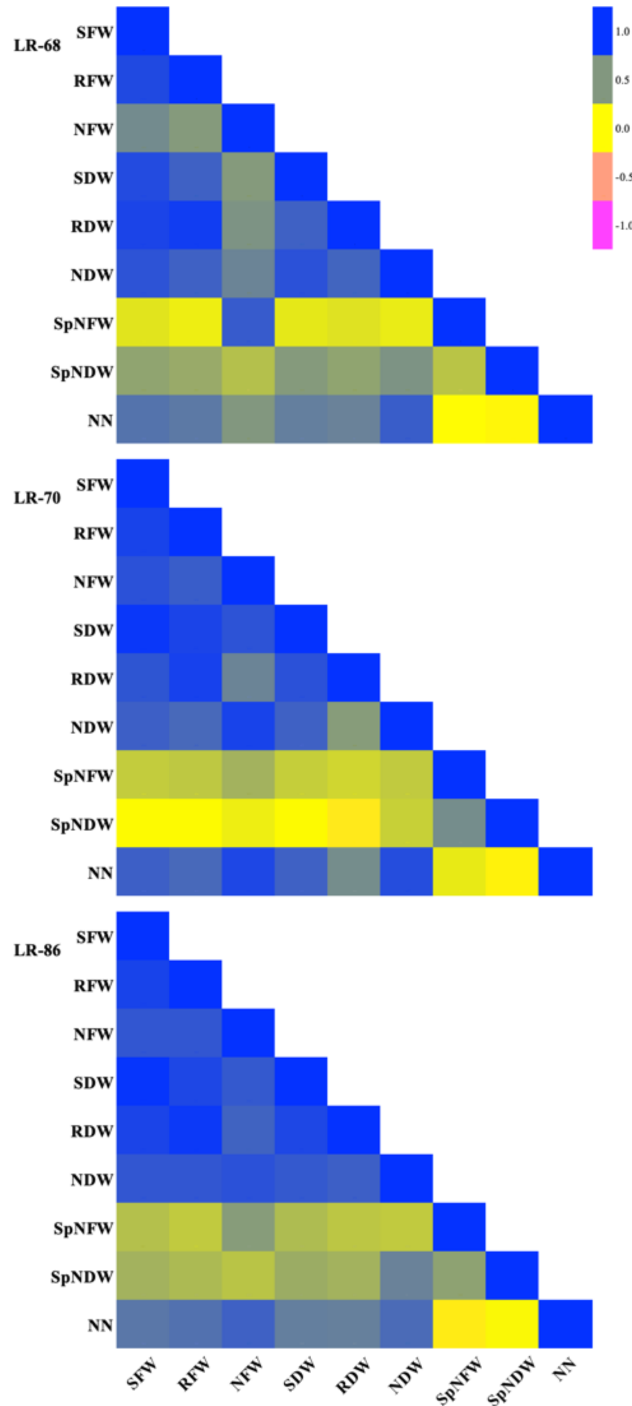

Figure 6.3. Phenotypic correlations among SFW: shoot fresh weight, RFW: root fresh weight, NFW: nodule fresh weight, SDW: shoot dry weight, RDW: root dry weight, NDW: nodule dry weight, SpNFW: specific nodule fresh weight, SpNDW: specific nodule dry weight and NN: number of nodules of RIL from the interspecific populations LR-68 (IG 72643 *L. orientalis* × CDC Greenstar *L. culinaris*), LR-70 (Eston *L. culinaris* × IG 72623 *L. odemensis*) and LR-86 (Lupa *L. culinaris* × BGE 016880 *L. orientalis*) inoculated with the strain NZLR-24 (*Rhizobium leguminosarum* bv. *viciae*) grown in a cabinet in plastic pouches and evaluated 30 DAS.

6.3.3. QTL for nodulation traits.

Three QTL for NN and one for SpNDW were identified in the LR-68 population (Table 6.1, Figure 6.4). The most significant markers for QTL associated with NN were mapped at 84.9 cM in LG1, 54.2 cM in LG3 and 46.4 cM in LG6. Combined, these three QTL explained 35.2% of the phenotypic variability. The most significant marker for SpNDW was mapped at 51.9 cM in LG7 and explained 14.7% of the phenotypic variance. Their additivity values reflected that the wild parent IG 72643 (*L. orientalis*) was the source of the positive (higher) allele for all four QTL.

Four QTL were mapped in the LR-70 population (Table 6.1, Figure 6.5). The most significant marker for SpNFW was mapped on LG 2 at 64.5 cM and explained 12.5% of the phenotypic variance. QTL for multiple traits co-localized on LG1, LG5 and LG6. The region spanning 38.5-49.6 cM of LG1 harboured significant QTL for both NFW and NDW and explained 15.7% and 14% of the phenotypic variance respectively. The region spanning 49.5-63.4 cM of LG5 contained another QTL for NFW as well as one for NN that explained 15.7 and 12.4% of the phenotypic variance respectively. Finally, the region on LG6 from 48.6-71.2 cM contained QTL for NFW, NDW and NN that explained 10.7, 11.6 and 5.6% of the phenotypic variance respectively. The cultivar Eston was the source of the positive (higher) allele for all QTL mapped in this population.

In the LR-86 population, eight QTL were mapped (Table 6.1, Figure 6.6). The region spanning 80.1-97.4 cM in LG1 harboured significant QTL for NFW and NDW that explained 3.6 and 4.0% of the phenotypic variance respectively. In LG1 there were also significant QTL for NFW, NDW, SpNFW and SpNDW in the region spanning 302.3-330.2 cM that explained 8.8, 9.3, 9.1 and 8.4% of the variation, respectively. The most significant marker for QTL *qSpNDW-7-1* was mapped at 187.0 cM in LG2 and explained 2.9% of the phenotypic variation. Four QTL were mapped in LG4: marker for two QTL for NDW were mapped at 62.7 and 87.8 cM that explained 4 and 8.1% of the phenotypic variation respectively. A third QTL for NFW had the most significant marker at 179.6 cM, explaining 7% of the variance. The last QTL in LG4 had the most significant marker at 489.3 cM explaining 22.5% of the phenotypic variance.

Table 6.1. Summary of QTLs identified for nodulation traits of RIL from the interspecific populations LR-68 (IG 72643 *L. orientalis* × CDC Greenstar *L. culinaris*), LR-70 (Eston *L. culinaris* × IG 72623 *L. odemensis*) and LR-86 (Lupa *L. culinaris* × BGE 016880 *L. orientalis*) inoculated with the strain NZLR-24 (*Rhizobium leguminosarum* bv. *viciae*) grown in a cabinet in plastic pouches and evaluated 30 DAS.

Population	QTL	Trait[a]	LG[b]	Position (cM)	Left marker	Right marker	Confidence interval (cM)	Peak LOD	%PVE[c]	Add[d]
LR68	*qNN-1*	NN	1	84.9	1p489488338	1p501804583	76.12-89.3	3.1	7.9	2.6
	qNN-3	NN	3	54.2	3p256015670	3p259089364	46.2-57.3	4.6	12.0	3.2
	qNN-6	NN	6	46.4	6p265329531	unitig3460p155559	39.3-49.6	5.7	15.3	3.7
	qSpNDW-7	SpNDW	7	51.9	7p31178036	7p32792357	43.7-57.4	4.5	14.7	0.0
LR-70	*qNFW-1*	NFW	1	42.7	1p310453504	1p329995358	38.5-49.6	3.1	15.7	19.2
	qNDW-1	NDW	1	42.8	1p310453504	1p329995358	41.2-44.5	6.0	14.0	3.6
	qSpNFW-2	SpNFW	2	64.5	2p485017602	2p486253806	41.5-68.2	3.0	12.5	0.2
	qNFW-5	NFW	5	58.3	5p448273968	5p455325800	49.5-63.4	4.4	15.7	30.3
	qNN-5	NN	5	56.1	5p446960616	5p452274789	50.4-61.3	10.1	12.4	22.2
	qNFW-6	NFW	6	64.1	6p350961800	6p351924311	54.3-72.2	5.1	10.7	25.2
	qNDW-6	NDW	6	64.1	6p350961800	6p351924311	54.3-62.5	5.0	11.6	3.6
	qNN-6	NN	6	62.2	6p350961800	6p351924311	48.6-61.2	6.1	5.6	14.4
LR86	*qNFW-1-1*	NFW	1	90.9	1p199191474	1p197082937	80.1-97.4	3.5	3.6	-24.0
	qNDW-1-1	NDW	1	90.6	1p199191474	1p197082937	80.1-96.2	3.7	4.0	-3.8
	qNFW-1-2	NFW	1	317.4	1p392586655	1p377699367	307.5-330.2	7.6	8.8	37.5
	qNDW-1-2	NDW	1	317.4	1p392586655	1p377699367	308.4-328.6	7.7	9.3	5.9
	qSpNFW-1	SpNFW	1	310.6	1p394661452	1p395094319	302.3-319.2	5.1	9.1	0.7
	qSpNDW-1	SpNDW	1	311.1	1p395094319	1p394365461	302.3-318.5	5.9	8.4	0.1
	qSpNDW-7	SpNDW	2	187.0	7p245246638	7p257290354	176.5-194.5	3.1	12.9	-0.1
	qNDW-4-1	NDW	4	62.7	4p16831524	4p15776900	59.3-68.3	6.7	4.0	5.4
	qNDW-4-2	NDW	4	87.8	4p25789506	4p26691244	79.4-89.6	11.6	8.1	-7.6
	qNFW-4	NFW	4	179.6	4p92262668	4p92262641	172.7-186.3	3.1	7.0	-24.2

Table 6.1. Continued.

qNN-4	NN	4	489.3	4p294499889	4p288403052	480.5-522.5	3.2	22.5	-6.0
qNFW-6	NFW	6	342.3	6p239525416	6p235883012	339.4-359.1	5.3	9.9	30.2
qNDW-6	NDW	6	342.3	6p239525416	6p235883012	339.4-349.8	4.1	11.0	4.2
qNN-6	NN	6	342.3	6p239525416	6p235883012	339.4-349.8	6.6	10.6	8.1

[c]%PVE: phenotypic variation explained by the marker. [d]Add: estimated additive effect of the marker. [b]LG: linkage group. [a]NFW: nodule fresh weight, NDW: nodule dry weight, SpNFW: specific nodule fresh weigth, SpNDW, specific nodule dry weight, NN: number of nodules. Alternated white-grey colors separate QTL with co-localized traits.

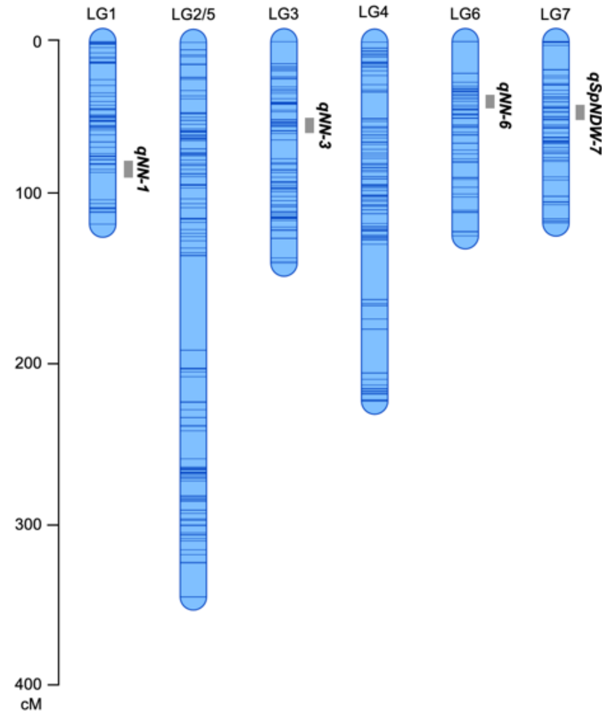

Figure 6.4. Distribution of QTL for nodulation traits in RIL of the interspecific population LR-68 (IG 72643 *L. orientalis* × CDC Greenstar *L. culinaris*), inoculated with the strain NZLR-24 (*Rhizobium leguminosarum* bv. *viciae*) grown in a cabinet in plastic pouches and evaluated 30 DAS. QTL are displayed on the right side of each linkage group with vertical bars showing the QTL confidence interval defined by 1-LOD drop.

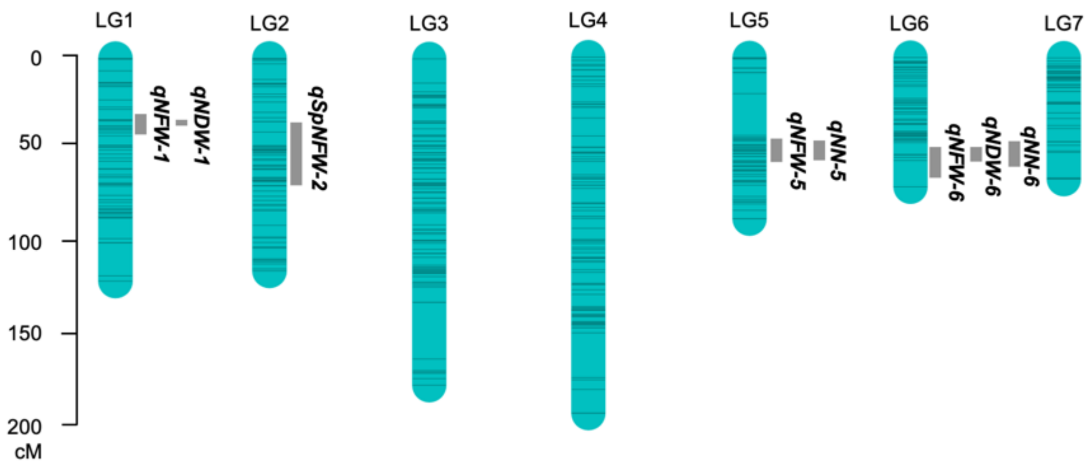

Figure 6.5. Distribution of QTL for nodulation traits in RIL of the interspecific population LR-70 (Eston *L. culinaris* × IG 72623 *L. odemensis*), inoculated with the strain NZLR-24 (*Rhizobium leguminosarum* bv. *viciae*) grown in a cabinet in plastic pouches and evaluated 30 DAS. QTL are displayed on the right side of each linkage group with vertical bars showing the QTL confidence interval defined by 1-LOD drop.

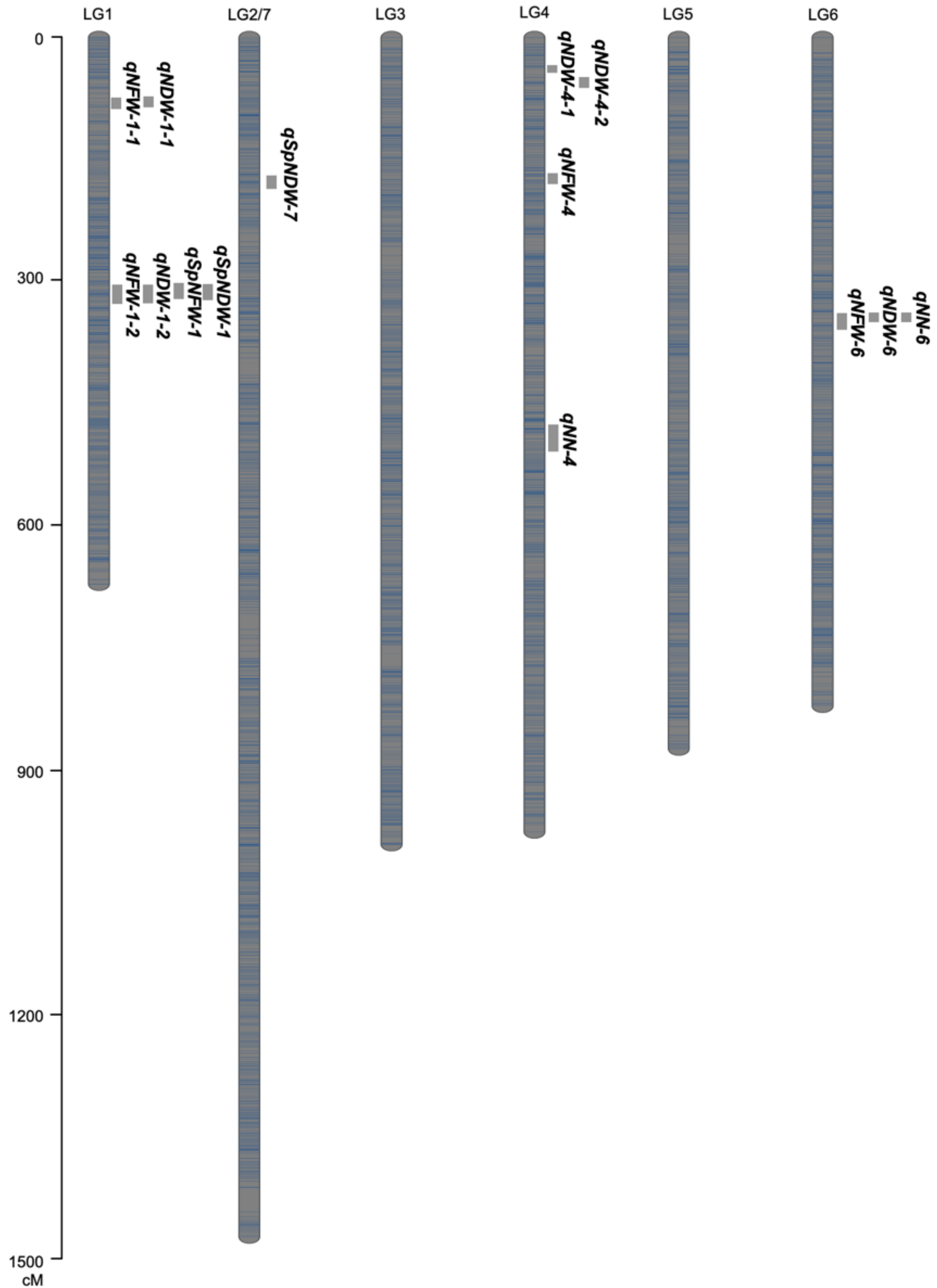

Figure 6.6. Distribution of QTL for nodulation traits in RIL of the interspecific population LR-86 (Lupa *L. culinaris* × BGE 016880 *L. orientalis*), inoculated with the strain NZLR-24 (*Rhizobium leguminosarum* bv. *viciae*) grown in a cabinet in plastic pouches and evaluated 30 DAS. QTL are displayed on the right side of each linkage group with vertical bars showing the QTL confidence interval defined by 1-LOD drop.

Lastly, the region spanning 339.4-359.1 cM in LG6 harboured QTL for NFW, NDW and NN, explaining 9.9, 11 and 10.6% of the phenotypic variance. The cultivar Lupa was the source of the positive (higher) allele for the QTL in the region spanning 301.4-330.2 cM in LG1 (NFW, NDW, SpNFW, SpNDW), NDW in LG4 and NFW, NDW and NN in LG6. BGE 016880 (*L. orientalis*) was the source of the positive (higher) allele for the QTL in the region spanning 80.1-97.4 cM in LG1 (NFW and NDW), SpNDW in LG2 and NFW, NDW and NN in LG4. QTL mapped for SFW, SDW, RFW, RDW are available in Table Y, Appendix Y.

6.3.3.1. Meta-QTL for nodulation traits.

There was strong overlap for QTL position and support intervals at two loci across all three populations: on chromosomes 1 and 6 (Figure 6.7 A and B). There was also overlapping QTL position and support interval on chromosome 7 for the populations LR-68 and LR-86 (Figure 6.7 C). In chromosomes 1 and 6 the overlapping regions had contrasting sources of the positive allele. From LR-68 the allelic source was IG 72643 *L. orientalis*, while in LR-70 and LR-86 the allelic sources were the *L. culinaris* parents Eston and Lupa. The positive allele sources of the region on chromosome 7 were the *L. orientalis* parents IG 72643 and BGE 016880 for the populations LR-68 and LR-86, respectively.

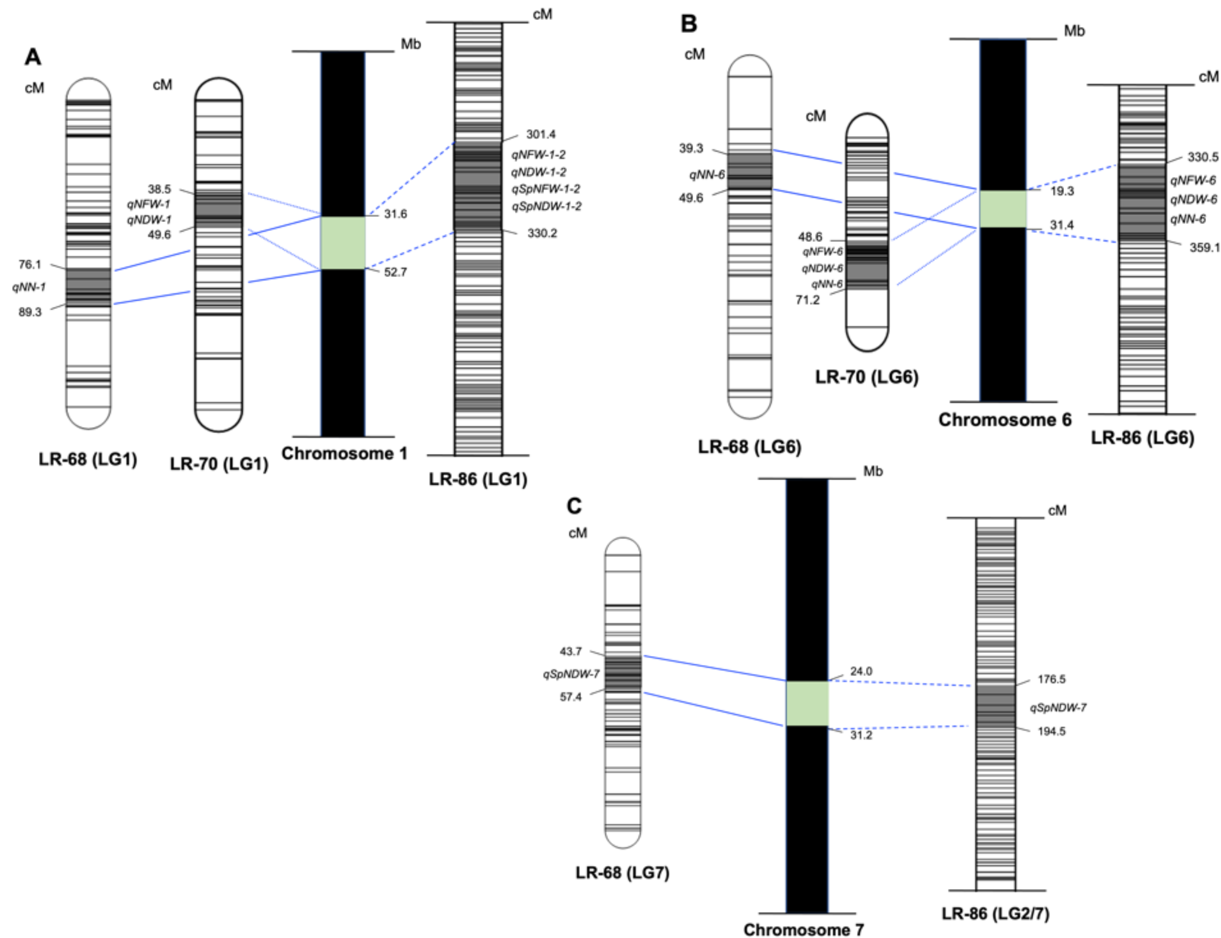

Figure 6.7. Linkage maps alignments for *Lens culinaris* chromosomes 1 (A) and 6 (B), with the corresponding linkage groups from LR-68 (IG 72643 *L. orientalis* × CDC Greenstar *L. culinaris*), LR-70 (Eston *L. culinaris* × IG 72623 *L. odemensis*) and LR-86 (Lupa *L. culinaris* × BGE 016880 *L. orientalis*) RIL populations. C: *Lens culinaris* chromosome 7 with the corresponding linkage groups from LR-68 and LR-86. QTL of N-fixation related phenotypes based on RILs inoculated with the strain NZLR-24 (*Rhizobium leguminosarum* bv. *viciae*) grown in a cabinet in plastic pouches and evaluated 30 DAS. Gray area represents the 1-LOD interval of confidence for each QTL. Linkage positions of markers used in each population aligned with their corresponding physical position in each chromosome, with the green lines showing the overlapping interval.

6.4. DISCUSSION

In this study, the interspecific populations LR-68, LR-70 and LR-86 were characterized for nitrogen fixation related traits following inoculation with the rhizobial strain NZLR24 (*Rlv*). These data were used to determine heritability and to gain a better understating of the genetic sources of variation for these characteristics, and the contribution from parents with contrasting N fixation ability from three *Lens* species. With wild germplasm being already part of the breeding program, and genotypic information of such germplasm available, it is possible and important to determine regions responsible for desirable and undesirable traits for the most characteristics possible, even when a specific trait is not one of the main objectives of the breeding program. One of these traits particularly important to legumes, is N fixation ability.

From the characteristics evaluated at flowering in Chapters 3 and 5, nodulation traits were clearly differentiated between the cultivated and wild groups and within wild accessions also. The focus was on these nodulation traits because the parental lines of the RIL populations have differential nodulation characteristics that affected their ability to fix N. All cultivars had determinate nodulation, varying mainly in the number of nodules. The three wild parents had different nodule phenotypes and also varied in number (Chapter 5). The parental lines of the LR-68 population, IG 72643 (*L. orientalis*) crossed with the cultivar CDC Greenstar, both exhibited desirable characteristics of N fixation. For instance, IG 72643 (*L. orientalis*) has palmate indeterminate nodulation which could be fixing N longer compared to CDC Greenstar. From the parental lines of the LR-70, both the cultivar Eston and the wild IG 72623 (*L. odemensis*), had intermediate N fixation ability. Both had determinate nodulation with IG 72623 (*L. odemensis*) being one of the few wilds exhibiting a nodule type similar to cultivars. From the LR-86 parental lines, the cultivar Lupa demonstrated low N fixing ability, while BGE 016880 (*L. orientalis*) was intermediate. BGE 016880 (*L. orientalis*) also had bifurcate palmate indeterminate nodules and the highest specific nodule weight when inoculated with NZLR-24 (*Rlv*) (Chapter 5). Cultivated parents varied in their number of nodules, CDC Greenstar, Eston and Lupa had high, intermediate and low number of nodules respectively. From the wild parents, IG 72643 (*L. orientalis*) and BGE 016880 (*L. orientalis*) had low numbers of nodules, while IG 72623 (*L. odemensis*) had higher numbers.

The LR-86 RIL population was the only one that exhibited transgressive segregation towards higher values of shoot, root and nodule weights. BGE 016880 *L. orientalis,* the donor wild parent of this population, had the highest specific nodule weight value in this experiment, consistent with the high values observed among the wild *Lens* species accessions studied earlier, which also had superior values of N fixed at flowering with the strain NZLR-24 (*Rlv*) (Chapter 5). Most of the H^2 values observed in this RIL population were intermediate, but not for specific nodule weight, which was low. Specific nodule weight is an indirect measure of the presence of indeterminate nodulation; however, heritability could improve if segregation of determinate vs indeterminate was analyzed directly.

The LR-68 and LR-86 populations were segregating for indeterminate nodules. One possibility to explain the low H^2 values for specific weights is that this phenotyping was conducted while indeterminate nodules were still increasing in size. Also, some lines could have been still developing more nodules in general in subsequent days. None of the cultivated parents have the NN observed before (Chapters 3 and 5). This suggests that the parents from these populations have a different speed of nodulation and this directly affected phenotypes obtained at 30 DAS.

The IG 72643 (*L. orientalis*) parent had a maturity delay of about a week when inoculated with NZLR-24 (*Rlv*) grown in the same chamber environment as this experiment (Chapter 5). Maturity was not measured in this study since plants were destructively sampled at flowering, but the anticipated maturity delay combined with its low specific weight in this experiment, suggests this genotype has prolonged nodulation activity after flowering and a different speed of nodulation compared to other accessions. Prolonged nodulation around flowering is desirable only if the plant can reach maturity without delays (Graham *et al*., 2004). The LR-68 population could be segregating for speed of nodulation and this directly affects the interpretation made at 30 DAS for phenotypes that were not yet fully expressed. Understanding this will be important to determine the contributions of the LR-68 population for better N fixation in lentils. The prolonged indeterminate nodulation post flowering in *Lens* accessions from different species had no maturity delays in most cases, except for a few accessions (Chapters 3 and 5).

The LR-70 RIL population had similar distributions of shoot and root weights to those observed in the LR-68 population, even though their parents exhibited different phenotypes. The H^2 values of the LR-70 were better, however. LR-70 had the greatest variation in NN and had individual

RILs with the highest NN. The wild parent, IG 72623 (*L. odemensis*) was phenotypically more similar to the cultivated parents than the other two wilds for nodulation traits, with similar NN and specific weight (Chapter 5, Table O, Appendix O), and the way determinate nodulation gives different characteristics can be observed in how this population had the highest correlation between NN and the rest of traits compared to the other two populations.

H^2 values for N fixation traits reported in other legume systems vary from 0.2-0.9 (Graham *et al.*, 2004). Typically, few progenies perform better than their parents when selecting for N fixation ability. The lowest values observed when selection was based on nodulation alone, and the best results obtained when characteristics of nodulation phenotypes, nodulation activity and enhanced yield were pyramided (Seido *et al.*, 2019; Graham *et al.*, 2004). Some characteristics will also be population specific, depending on their parents' genotypes, and different genes are likely segregating in different populations, as observed in this experiment. With very diverse lentil germplasm, the correlations among nodulation, N fixation at flowering and seed yield were intermediate (Chapter 3), therefore, if these RIL populations were studied for N accumulation parameters, nodule activity or seed yield, results might reveal other important determining characteristics and H^2 values would increase as a result. COVID restrictions in place at the time of this experiment prevented these additional parameters from being investigated and I strongly recommend following up when possible.

QTL mapping of nodulation traits helps resolve the genetics underlying the differential nodulations systems observed in wild and cultivated lentils. This knowledge will provide a better capacity for selecting what regions to keep following interspecies hybridization and reduce linkage drag when introgressing wild germplasm into the breeding program. Translocations and inversions in one genome relative to another lead to reduced recombination and pseudo-linkage in genetic linkage maps. Reduced recombination among *Lens* interspecific populations has been observed, which leads to linkage drag of potentially undesirable blocks of genes (Bett 2021, personal communication). Pseudo-linkage results in markers from two separate chromosomes to map together. In the LR-68 linkage map, chromosomes 2 and 5 are pseudo-linked. In the LR-86 linkage map, chromosomes 2 and 7 and pseudo-linked. Reduced recombination increases the region of the physical genome covered by a QTL, making candidate gene identification more

difficult than usual. None of the QTL were mapped in pseudo-linked chromosomes in *L. orientalis* which could have limited the ability to continue to work with this populations.

There were QTL hotspots on chromosomes 1,6, and 7 that appear across the 3 populations (Figure 6.7). These were identified using multiple traits and help explain the correlations observed among them and demonstrates the power of mapping multiple populations. Consensus regions reduced the physical intervals identified in individual populations and will facilitate the identification of candidate genes. These meta-QTL suggest that different nodule and plant characteristics that are important for N fixation are controlled by few regions of the genome. On chromosome 6, the overlapping QTL in the LR-70 and LR-86 populations are related to nodule number, nodule weight and specific nodule weight. NN was at that same region in LR-68 but not the other measures, supporting the argument that this population could be segregating for speed of nodulation and even though nodules were present, they had not reached their full size by the time they were sampled. This is further supported by the fact that IG 72643 (*L. orientalis*) was the allelic source of the positive variation.

The meta-QTL on chromosome 1 co-localized NN in the LR-68 population, nodule weight in LR-70 and nodule weight and specific nodule weight in LR-86. Again, with nodule phenotypic diversity it is necessary to take into consideration several characteristics. Even though they correlated in general, correlations were population specific. LR-70 had the lowest correlation among specific weights with the rest of nodulation traits, because of the characteristics of the parental lines, and NN had the strongest correlation and was in fact the only trait that mapped here. The third meta-QTL, on chromosome 7, was identified in the LR-68 and LR-86 populations only for SpNDW and did not co-localized with the QTL for SpNFW. *L. orientalis* was the positive allelic source in both populations and was not expected to be mapped in LR-70 where both parents have lower specific nodule weights. This overlapping region on chromosome 7 is key for understanding higher specific nodule weight in *L. orientalis*.

Co-localized QTL for N fixation related traits have been reported in other legume systems. In *Lotus japonicus* symbiotic nodulation activity traits also co-localized and were established to be determined by the same locus (Tominaga *et al*., 2012). In cowpea, inefficient nodulation and N fixation traits were co-localized in a single QTL with major effects (Ohlson *et al*., 2018). In the

LR-86 population, a population-specific QTL was mapped for NFW that did not co-localized with any other trait; this was also reported in cowpea (Olhson *et al*., 2018). In common bean, N fixation traits (Ndfa, N concentration, N yield) co-localized with percentage seed N and seed yield (Heilig *et al*., 2017). How the nodulation traits mapped in this study are co-localized with nodule activity and enhanced yield via N fixation as well as protein in seed will be key for determining the utility of markers associated with nodulation traits.

In addition to the meta-QTL mapped, there are population-specific QTL mapped that are worth looking at for understanding specific parental combinations. These population specific QTL could be regions that are fixed in the other interspecific populations or there was simply insufficient within population variation accurately phenotype and map those regions as was the case of specific nodule weight in LR-68. The population-specific QTL identified varied in the amount of phenotypic variance they explained and the number of traits that were controlled by those regions of the genome. The only QTL mapped on chromosome 3 was in LR-68 for number of nodules and explained 12% of the phenotypic variation. LR-70 had unique QTL on LG2, explaining 12.5% of the variation in specific nodule weight, and on LG5 for nodule fresh weight and number of nodules, explaining 15.7 and 12.4% of the phenotypic variance, respectively. This population had smaller nodules and higher number of nodules compared to the other two populations, and this unique QTL helps explain this phenotype.

LR-86 had four QTL on LG4. As discussed in previous sections, this population had higher specific nodule weight and fewer nodules compared to the other two populations before flowering. Three QTL had IG 72643 (*L. orientalis*) as the positive allelic source - for NDW, NFW and NN, and explained 8.1, 7.0 and 22.5% of the phenotypic variation. The fourth QTL, *qNDW-4-1*, was related to nodule dry weight specifically, explaining 4% of the phenotypic variance and with the larger weights coming from the cultivated parent allele. NFW and NDW were mapped separately in this population identifying different regions, confirming the utility of measuring both in populations in which diverse nodule phenotypes are segregating. The unique QTL mapped in LR-86 could be key for understanding early indeterminate nodulation in lentil, that was mapped using specific nodule weight.

The segregation of desirable phenotypes in the LR-86 population and the identification of QTL from the wild parent BGE 016880 (*L. orientalis*) responsible for differentiated higher specific nodule weight represent a key resource for understanding introgression of this characteristic. Breeding for cultivars with high number of small nodules may eventually compromise yield because the additional nodules compete with the energy required for high seed production (Graham *et al*., 2004). Observations in Chapter 3 and 5 suggest that most lentil cultivars cannot express their full yield potential under symbiosis. Bigger indeterminate nodule phenotypes that are quickly developed could represent a crucial characteristic to reintroduce from the wilds. Introgression of this characteristic through interspecific hybridization could help break this barrier and reintroduce this ability into lentil cultivars.

Wilds can be a source of useful variation, but just like when selecting cultivated parents, it requires cautious parental selection, even more so with the known reduced recombination. Sources of variation depended on the population in each of these three populations. Both *L. orientalis* parents were the allelic source on their populations, however *L. odemensis* parent of LR-70 was not. As the population whose allelic sources weas the *L. culinaris* parent, Eston, the LR-70 population gives an insight into the specific alleles that might be important to keep from *L. culinaris* in future interspecific crosses. Specific QTL mapped in the LR-70 population could also be an important resource for understanding regions responsible for higher NN. These findings suggests that breeding for nodulation should focus on plant phenotypes with the fewest nodules that most effectively fix N as determined by higher specific nodule weight.

Speed of nodulation has to be determined in any future study, because it is crucial for legumes to reach maturity on time in the Northern Great Plains. How this affects flowering and maturity in lines with desirable characteristics has to be determined under field conditions, however. The overall contributions of CDC Greenstar to N fixation should be determined. As a cultivar, it is a valuable resource for its high yielding capacity under symbiosis, something not observed in any other cultivar tested (Chapter 3 and 5). At the same time, it will also be important to determine its ability in intraspecific populations, using populations in which CDC Greenstar is the female parent as well as with other wild donor parents like BGE 016880 (*L. orientalis*).

The specific nodule weight phenotype was an indirect measure of determinate vs determinate nodule phenotypes. However, it is important to determine the segregation of these two nodule phenotypes directly and confirm this relationship. Photographs were taken for each experimental unit but have not been investigated closely because of limitations in time for phenotyping, given that this whole chapter was conducted under COVID-19 restrictions.

These QTL contribute to the understanding of the genetics underlaying these traits and facilitate the identification of candidate genes. Given that these experiments were conducted in interspecific populations, it also helps to build our knowledge on the potential for introgression from exotic germplasm and the regions to keep when breeding for better N fixation ability.

Co-localization of the mapped traits was expected given the high correlation among phenotypic traits. How the nodule phenotypic traits mapped in this study are co-localized with N fixation, and N yield in lentil will be crucial to determine, because transgressive segregation towards better BNF will require pyramiding genes for desirable nodule phenotypes, nodule activity, speed of nodulation, and N and seed yield.

7. GENERAL DISCUSSION

7.1. Research summary

The purpose of this whole study was to establish the roles that diverse genetic resources of both *Lens* and *Rhizobium* species might play in the breeding of lentil for better nitrogen fixation ability. It was also intended to establish the range of infectiveness and effectiveness based on the interactions of multiple combinations of both organisms. Furthermore, initial steps towards establishing the genetic variation contributing to infectiveness and effectiveness were made with a view to identifying regions of the genome (QTL) responsible for nodulation traits. Before this, little was known about the genetics of N fixation in lentil and there were definitely no QTL related to N fixation characteristics available to help breeders.

It was hypothesized that reaching to wider genetic resources of both *Lens* and *Rhizobium* would allow the identification of superior individuals for improving N fixation in lentils. In Chapter 3, contrasting N fixation related abilities were observed within the genus *Lens,* with genotypes with desirable characteristics from both cultivated and wild pools and no particular species standing out. Genotypes like CDC Greenstar and IG 72643 (*L. orientalis*) had a generally desirable symbiotic ability based on their response in a number of the traits measured, including N fixation and seed yield in symbiosis with a commercial strain. Wild accessions showed additional characteristics of interest for further exploration such as indeterminate nodulation and stable yield when inoculated compared to fertilized with synthetic N, as well as root adaptation mechanisms to efficiently acquire N and higher seed protein concentration compared to cultivars. Findings from field experimentation with multiple diverse *Rhizobium* species (Chapter 4) confirmed that there are more suitable strain resources for inoculum in the Northern Great Plains. CDC Maxim inoculated with the strain NZLR-24 (*Rlv*) yielded 9% higher compared to when inoculated with commercial inoculant BASF 1435 (*Rlv*) and 15% more than when not inoculated at all. None of the strains resulted in yields less than with the commercial one and represent genetic diversity that could be useful for more resilient inoculum across stressful environments.

It was also hypothesized that there would be specificity at the accession × strain level and defining the restrictions this would have on the infectiveness and effectiveness of these superior resources was important for future work. Interactions were indeed observed at the accession ×

strain level, and some relationships were very specific when it came to effective infection and N fixation. Wild *Lens* accessions were, in general, very promiscuous, and the strain NZLR-24 (*Rlv*) was demonstrated to be as effective with a greater range on *Lens* as it was under field conditions with the cultivar CDC Maxim.

Lastly, it was hypothesized that characterizing N fixation related traits in RILs from interspecific populations of parents with contrasting N fixation ability, would help elucidate the inheritance of these traits. Additionally, QTL analysis of nodulation traits would lead to mapping regions of the lentil genome responsible for diverse phenotypic nodulation in three *Lens* species and establish their allelic contribution to BNF. Transgressive segregation towards higher phenotypes was observed in the LR-86 population for several N fixation related traits. Sixteen QTL were mapped across the three interspecific populations for diverse traits. QTL overlapped across all three populations on chromosomes 1 and 6: co-localizing multiple different nodulation traits. An interesting QTL for specific nodule weight with *L. orientalis* as the allelic source, was mapped in both LR-68 and LR-86 on chromosome 7 and warrants further investigation.

7.2. N fixation in lentils: why should we breed for better BNF?

Contributions of the BNF process to lentil productivity have been measured and reviewed in the past. Selection of superior strains, lentil genotypes, and combinations of these have been reported in the literature in studies conducted 15-20 years ago (Moawad and Beck 1991; Unkovich and Pate 2000; Haffez *et al*., 2000; Martinez-Romero 2003; Bremer *et al*., 1990; Abi-Ghanem *et al*., 2011) but not much seems to have happened since. A publication in Nature reported the scarcity of breeding efforts in this field (Knight 2003) and 18 years later no substantial progress has occurred for most legumes, including lentil. It has been proposed that a second green revolution based on agro-environmental resilience rather than application of more synthetic fertilizers should boost crops yields and increase food security, and root adaptations and enhanced symbiotic ability should be a fundamental breeding objective for the future (Lynch 2007).

The very first challenge is not improving BNF, it is making sure to maintain a symbiotic relationship that does not negatively affect the crop performance. As beneficial as BNF can be, it is possible to have undesirable effects as a product of deficient symbiosis. Within the context of this study, it was established that such suboptimal relationships in lentil can delay flowering and alter days to maturity. N fixation is usually related to altered flowering time, but in the Northern

Great Plains, overall time to maturity is also big constraint. It was also established that seed percentage protein and protein yield, which are critical for a protein crop, are highly affected by this relationship.

Cultivar differential ability for N fixation has been demonstrated in all crop legume systems. All CDC cultivars evaluated in this study showed more desirable symbiotic characteristics than those cultivars selected elsewhere under different selection environments. This suggest that they have been indirectly selected in the presence of the local resident population for BNF and have an overall superior ability under local conditions. However, significant differences in symbiotic ability were established even among the CDC lines for several characteristics. In addition, findings from this study suggest that the traits we have been using for selection of superior genotypes, or have considered desirable, might actually be counterproductive for overall crop performance with respect to BNF. As found here, and previously suggested in literature in other legume systems, the nodulation structure we have been selecting for is probably not the most adequate for enhancing BNF. A good example of this is the results for seed yield per plant observed in Chapter 3, for the cultivars CDC Maxim and CDC Greenstar. Both cultivars yielded about the same and had 450 and 301 nodules per plant, respectively. The additional 150 nodules in CDC Maxim apparently did not contribute to the plant productivity. The inefficiency of cultivars with higher NN was proposed over 50 years ago (Nutman 1967), but many evaluation systems use scales of nodulation that select for higher numbers of nodules. It will be important to evaluate this relationship in more detail in the interspecific RIL populations with contrasting phenotypic nodulation so that we can better understand the underlying genetics.

Breeding will play a determining role in taking this relationship in lentil to the level needed for maximum crop performance now and in the future. Understanding it probably should be part of all legume breeding programs. Breeding methods used in self-pollinating crops have been used in breeding for improved BNF, including simple, double and three-way crosses, backcross-inbred and recurrent selection (Graham *et al*., 2004). Like any other characteristic to be enhanced, the first step involves evaluating diverse germplasm and defining which traits will help to enhance BNF. Determining the genetic variation available, identification of parents differing in these traits, understanding the trait genetics, and developing a breeding strategy that includes a simple and affordable field-based method for selection are critical. For the lentil-

Rhizobium relationship the breeding strategy should also include the study of lentil × *Rhizobium* interactions, while considering the effects of competitively inefficient microbes present in the soil and validating superior relationships in the presence of stresses in the local target environment. As a contribution to this workflow, diverse representative germplasm of *Lens* species were evaluated in this study, identifying potentially useful parental lines. A better understanding of the most significant traits to measure and evaluation of the genetic variation of some of these traits in populations, as well as identifying genetic regions responsible for nodulation traits in three of the *Lens* species is important. The sections below will further elucidate they ways in which this work has contributed to breeding for better BNF, including the potential use of related wild *Lens* species.

7.2.1. Will going back to wilds benefit breeding for BNF?

Yes, there are characteristics of interest in wilds for breeding for better BNF ability. With no doubt, lentil cultivars have pyramided genes that makes them succeed under current environmental conditions and are genetically advanced for seed characteristics and yield. However, their narrow genetic base and how this phenomenon has impacted the efficiency for BNF is reflected in the findings of this study. Additionally, wild *Lens* species are already a systematic part of the breeding program for different traits other than BNF. Deploying the full potential of wild germplasm requires establishing the genetic regions responsible for multiple traits of interest, even if not breeding directly for those characteristics, to make decisions on the regions to keep and those to avoid in the process of re-introducing wild genetics to the cultivated gene pool.

One evident characteristic is the indeterminate nodulation found in all wild *Lens* species which was responsible for the production of phenotypes with more stable yields and higher protein concentration, in contrast to the high counts of small nodules observed in cultivars. This is an important characteristic to regain from wild species since it seems to no longer be present in cultivars. Continuing the breeding of high yielding varieties for enhanced BNF with the current characteristics of nodulation in cultivars will lead to competition between seed and nodules for energy, limiting progress. Regions related to higher specific nodule weight mapped on chromosome 7, unique to *L. orientalis* in LR-68 and LR-86, could be a starting point for understanding were to begin regaining this characteristic. How will high yielding lentil cultivars

do with interspecific nodulation? Will that be more sustainable in the long-term? The findings of this study suggest that redesigning the nodulation system in cultivated lentil to a minimal indeterminate, and more efficient nodulation system could be a more sustainable approach.
The other differentiated trait observed was the higher seed protein percentage concentration observed in wilds, which was even higher when these accessions were inoculated. The high protein content and protein yield observed for inoculated wilds could be key in breeding for this characteristic and should be followed up on.

7.3. What are the relevant traits to measure in *Lens* for BNF?

In the process of measuring BNF traits, some conclusions can be made about what is worth measuring when evaluating such types of diverse germplasm. It is not a straightforward route to compare quantitative abilities between cultivated and wild types at any level because most differences are simply because they are inherently different plants. There is no literature in how and what to measure in these cases. A publication in soybean (*Glycine max*) (Munoz *et al*., 2016) comparing cultivars with accession of its wild relative *G. soja* suggested that cultivars gained more fixation ability based on measurements of nodule number, nodule fresh weight, total ureides and plant fresh weight. Although these cover aspects of nodulation phenotypes and nodule activity, there are limitations to discriminating N fixation related abilities based merely on these traits.

The literature is clear in stating that there is no right or wrong way to measure BNF, and it all depends on the goals of the program. Most importantly there has to be a balance between precision and practicality, and methods should include assessment of nodulation, net values of N accumulated, nodulation activity and grain productivity (Unkovich *et al*., 2008). In my work the overall goal was to determine if wild *Lens* species had something to contribute to cultivated lentil, because the wilds were expected to carry desirable genes for a number of different traits, therefore, several approaches were used. Three of the four methods suggested by Unkovich *et al*., 2008 were covered in this work:
- assessment of nodulation characteristics, including different nodule phenotypic parameters
- net increases of N that were determined by partitioning with the difference method and as a total amount with the N balance method.
- translocation of N that was estimated with harvest index, seed yield and protein content.

The assessment of nodulation included direct nodule traits such as nodule number (NN), nodule dry weight (NDW) and nodule fresh weight (NFW). From these, specific nodule weight was estimated for nodule size. Having both NFW and NDW was important because of the differences in nodule size as confirmed in Chapters 5 and 6, and they were not always strongly correlated. Values of N fixed were estimated with the N difference method with the full set of selected wilds inoculated with the commercial strain BASF 1435 (*Rlv*). Again, using the N balance method, a smaller group of accessions combined with 5 *Rhizobium* strains were tested. With the N difference method, it was possible to explore additional information related to the different root phenotypes of these accessions and how they related to BNF and the ability to accumulate N under the presence of different sources and amounts of available N. With both methods, shoot dry weight (SDW), root dry weight (RDW), root architecture measured with WinRhizo©, N concentration in shoots, SPAD, and chlorophyl measured with PhotosynQ© were analyzed to determine the effects of the symbiotic relationships. Shoot and root parameters were traits used to determine N fixation and N accumulation, but they were not compared between wilds and cultivars, because they were expected to be different. From the nodule and shoot measurements, an estimate of efficiency can be calculated as N_2 fixed in shoots per mg of nodule weight. SPAD and PhotosynQ© measurements turned out to be inaccurate because of the small size of lentil leaves, therefore, were not used for interpretation in this study. They did, however, help established the limitations for their use in *Lens* species which will be discussed below. Productivity measurements included yield, seed percentage protein, protein yield and harvest index and all contributed to the understanding of differential abilities in N fixation across the plant and *Rhizobium* genotypes.

These findings validated the need to measure several phenotypic characteristics and with different methods, under different conditions if possible. When selecting parental lines, especially from very diverse germplasm, there is no magic trait; the greater number of traits observed, the higher the ability to discover novel phenotypes. If selecting progeny, fewer characteristics need to be looked at, i.e., those contrasting in their parents. In all three populations evaluated for the QTL study (Chapter 6), shoot weights strongly correlated to RDW and NDW, meaning one can be used as a good predictor of the others, simplifying phenotyping. It has been suggested that selection of parents that contribute to higher transgressive segregation

should consider nodule mass per plant, their activity, duration on the plant, and how they impact the plant cycle and nutrient translocation (Graham 2004). When evaluating progeny, especially in the field, yield, biomass and N accumulation at flowering could be sufficient, but selection based only on yield alone likely will lead to a loss of BNF ability (Graham 2004). Can breeding programs engage in highly demanding phenotyping? Not really but breeding for better symbiosis should be part of all legume breeding programs. Two key advances need to be conquered in order to make BNF more attractive to breeders: affordable and practical phenotyping techniques and tools for marker-assisted selection. Neither of which has been readily available.

7.3.1. Limitations of this study

The architecture of lentil leaves presents a challenge for some phenotyping methods. Non-destructive measures of overall plant health that are widely available need to be optimized for lentils, and other small-leaved crops in general. It would be convenient to have a reliable technique of measuring plant health that is highly correlated to N concentration for indirect selection in a non-invasive, non-destructive way. The usual pieces of equipment to do this, such as SPAD meters are very sensitive, however, and if one leaf is not big enough to cover the reading area, or if leaflets overlap, readings are incorrect. Potential alternatives will be discussed under future work (section 7.6).

All data obtained under controlled environment conditions will have limitations and the results are often limited to the specific conditions of the experiment. For this reason, it is important to validate a genotype's N fixation related abilities using more than one method. Coefficients of correlation changed to some degree based on the different genetic diversity evaluated in each experiment, but also as a product of the way plants adapted to the specific conditions under which they were evaluated. See Chapter 3 for a more detailed discussion. Some of these differences existed across experiments, however, some traits were more consistent, including seed weight, which had a very strong correlation to plant weight and a moderate correlation to nodulation parameters in the accession × strain experiment (Chapter 5). This experiment was conducted under the most controlled of conditions, and with N fixation as the only N source. In contrast, correlations were lower between seed weight and plant weights, but stronger between seed weight and nodulation, in the first experiment with only one strain (Chapter 3). In Chapter 3

the diversity among *Lens* was greater compared to the rest of the experiments and there was less control of the environment compared to the experiment described in Chapter 5.

Correlations between nodulation and plant weight in the RILs (Chapter 6) were population-specific and were best explained by the contrasting characteristics of their parents. For example, specific nodule weights were correlated to NFW, NDW or the rest of the plant weights according to the size of the nodules of their parents. These correlations were not observed across the other experiments with much more diverse germplasm. This confirms the importance of understanding which parameters are functioning differentially in the specific set of germplasm under study, and to select the experimental set-up and design accordingly. Extrapolating data evaluated under controlled conditions will always have some limitations, especially for a quantitative trait that involves the relationship of two factors and their complex interactions.

7.3.2. The need for new affordable and practical technology to assess BNF.

Phenotyping a complete plant for a number of traits demands intense labor, especially when this involves root washing. About 20 plants per day were processed for the experiments in Chapters 3 and 5 at a very good pace. About 105 days were spent only washing roots and phenotyping nodules from plants established in pots or tubes in these two experiments. If breeding for BNF will be part of the lentil breeding program, this is clearly not an option and non-destructive techniques are necessary to facilitate the process. Only then would it become more attractive for programs to adopt a systematic screening process.

Much of these techniques haven't changed much since the 1980's. This has a lot to do with the tendencies in research for BNF. There was substantial research in the 1980's and 90's for BNF in breeding programs, and this field lost importance with the availability of synthetic fertilizers. For 20 years, breeding did not consider microbial relations nor root systems for the most part. Recently, the role that symbiotic processes can play in preparing our crops for current and future demands is being better acknowledge and also better understood with modern tools. In this context, it is necessary that phenotyping systems be updated according to the new resources now available.

There are widely used techniques like WinRhizo© that measure root parameters and can automatically count nodules, but it is 2-dimensional and still requires root washing. In the last 5

years, non-destructive tools for evaluating root systems have become available, such as Minirhizotron© tubes, that consist of cameras placed inside a tube that is inserted in the soil. Rhizocam (Rahman *et al*., 2020) was reported as an alternative tool with more power of operation and a more affordable price compared to Minirhizotrons©. Also, a position emission tomography (PET) detector (BioPET) was installed in the Cyclotron Sciences facility at the U of S (Teymurazyan *et al*., 2017) which can image roots in 3D. These technologies have been developed for studying root systems and need to be optimized for *in vivo* evaluation of nodulation. They could become useful in the near future for chamber and-field based studies. A cheaper image-based phenotyping solution based on growing plants in plastic pouches has been validated in peas (*Pisum sativum*) for studies of nodulation (Remmler *et al*., 2014) for rapid evaluation of nodulation and nodule-root relations. This system was used for evaluating the three RIL populations (Chapter 6), and image-based analysis will be conducted from those studies for publication.

A measurement system for hydrogenase activity is also available for evaluating N fixation activity, although it requires the development of *Hup*$^{-}$ mutants and sealed tubes to conduct the measurements. A further limitation of this technique is that it measures activity at a specific time-point and in order to produce sequential data, requires sequential phenotyping that could also affect the plant, altering the phenotype.

Screening of progeny for better BNF has focused mostly on yield, biomass and N content in the shoot (Graham *et al*., 2004) which is too crude to get at the finer differences that could be pyramided to lead to significant improvements. With modern tools, this doesn't have to be the case, as more parameters could be extracted from *in vivo* sequential measurements in field experimentation. Integrating these technologies into the lentil breeding program will be key for assessing selection for better BNF ability and tracking phenotypic root traits in general. The present study generated information that will be used for subsequent analysis of imaged-based techniques and will be discussed under future work (section 7.6).

7.4. Optimizing BNF would directly benefit producers in the Northern Great Plains.

Any breeding for better BNF should be done with the best inoculants available. Diverse strains that nodulate lentils have recently become available (Gai *et al*., 2021). To decide which to proceed with it was necessary to assess their activity under local conditions. The superior strain

identified, NZLR-24 (*Rlv*), represents a resource that could benefit the cropping system in the Northern Great Plains.

Oilseed and grain farming had the highest operating profits, mainly in SK and MB, compared to all other agricultural activities in Canada (STATCAN 2020). These are average values per province, but as reviewed in Chapter 2.3.3, the current direct effects of %Ndfa in this area can be less than 10% in some farming systems. Within the context of this study, just by applying a different source of inoculum, for instance with the strain NZLR-24 (*Rlv*), CDC Maxim yielded 15% (2894.06 kg ha^{-1}) more compared to non-inoculated and 9% (2507.91 kg ha^{-1}) more compared to inoculation with the commercial strain BASF 1435 (*Rlv*) (2612.42 kg ha^{-1}). According to STATCAN, the average Canadian yield of lentil in 2020 was 1.460 t ha^{-1} with an average price of 465 dollars per t. Using this average as reference, and the increases in yield observed in this experiment (Chapter 4), a farmer growing CDC Maxim but not inoculating could observe an increase in profits of 316 dollars per ha, and a farmer currently using the BASF 1435 (*Rlv*) inoculant, could obtain an increase in profits of 190 dollars per ha, by switching to an inoculant with the strain NZLR-24 (*Rlv*).

This research also supports the importance of using inoculants even when there is an established population of rhizobia in the soil. Revising the constituent strains of inoculants will contribute to maximizing the full potential of modern varieties and can make significant differences in profits. It is important to note that BNF plays a key role in agriculture in the Northern Great Plains, not only increasing profits from lentil yields, but also with established benefits to subsequent cereals and oilseeds in the cropping system.

7.5. Conclusions

These studies delivered several components that are a good starting point for breeding for better BNF in lentils - defining the role wild introgression can play for recovering specific BNF related characteristics no longer present in cultivars, establishing potential suitable parents, as well as identifying key traits to be evaluated. It validated the utility of revising new available strain resources under local conditions. It also provided a better understanding of the genetic variation of such traits and reported the first group of QTL responsible for nodulation traits in lentils.

This is a very small part of what is needs to be done to enhance BNF in the lentil crop for sustainability. Enhancing N fixation will be the smartest route to produce plant protein with a minimal environmental footprint. We will need to redesign the root system of lentils, which as shown in Chapter 3, is highly affected by established symbiotic relationships. As a consequence, this will require that the root systems are able to support efficient symbiotic structures without affecting acquisition of other nutrients.

Equally important for the success of breeding for this trait will be integrating the knowledge gained in other related disciplines in the same way that is happening for other traits. In the last 10 years, a significant gain in knowledge has happened related to the signaling between host-legume and symbionts, as well as nodule activity and bacterial fitness inside the nodule, methods for tracking strain occupancy, and widely used molecular tools. A collaborative effort among multiple disciplines will produce the best results for improving BNF.

7.6. Future work

Given the complex nature of the BNF process and the multiple factors that influence this relationship, a wide range of opportunities for further learning about this process in lentil exist at different levels based on the findings of this study. This includes studies closely related to the data directly and indirectly generated and not analyzed within the context of this study, as well as potential subsequent experimentation directly related to these findings.

7.6.1. Additional data from this study.

This work generated additional information that was not presented in this thesis and is worth further exploration. As mentioned in Chapter 3, measurements with SPAD and PhotosynQ© meters were taken at flowering that were not used. Simultaneously, RGB images of leaves were taken. Features from those images could be extracted and analyzed to determine if and how they correlate to N concentration in shoots. This could validate the utility of image-based measurements as an indirect measure of N concentration.

From Chapter 3 and 5, novel nodule phenotypes were observed. As genome sequences of all the wild *Lens* species will soon be available, comparison could be made among assemblies to explore the genetic basis of these phenotypic variation. This kind of investigation would be facilitated with available orthologs from *Medicago truncatulata* and other sequenced legumes.

In the study described in Chapter 6, all experimental units were imaged twice – once at 15 DAS and a second time at 30 DAS. This represents a valuable source for extracting features from images that were measured manually to validate image-based phenotyping techniques, as well as to extract other possible features related to root architecture. Evaluation of the BNF of individuals using this system has been successful in pea (*Pisum sativum*) (Remmler *et al*., 2014). Additionally, comparisons can be made from the two pictures taken from each individual to determine speed of nodulation, which was not measure in this study and could be an important factor in understanding the nature of desirable N fixation observations.

The role of indeterminate nodulation in the higher values of seed percentage protein found in wilds could also be important to assess. One approach could be evaluating seed percentage protein and protein yield of RILs from the interspecific populations LR-68 and LR-86 with contrasting nodule phenotypes.

7.6.2. Subsequent experimentation

Given the range of phenotypic characteristics displayed by all *Lens* species, one approach could be conducting a genome-wide association study (GWAS) on a greater set of both cultivated and wilds. In Chapters 3 and 5, genotype-specific characteristics of interest were found that did not belong to any particular species. Association mapping could help reveal the genetic basis of phenotypic nodulation diversity in lentils as well as other BNF related traits of interest.

CDC Greenstar stood out as a valuable source for breeding for better BNF based on it having several desirable nitrogen fixing characteristics. Studies of intra and interspecific populations with CDC Greenstar as a parent could better establish its contributions using populations that have already been developed, such as LR-104.

There are three *Rlv* strains closely related to NZLR-24 (*Rlv*) (Gai *et al*., 2021), which could be valuable to test to determine if the high seed yielding results observed with NZLR-24 (*Rlv*) are a feature of this group in general or are unique to this strain. There are also 30 wild-type strains from the group of Oyali B (*Rlv*), which is notable for its stability under local conditions, ability to efficiently nodulate diverse *Lens* and for the resilience characteristic of these strains to tolerate changing and limiting agro-environmental conditions (Zahran *et al*., 2013). Exploring co-inoculations of strains among the groups with efficient performance under field conditions could

be another approach for producing superior inoculants. This is a technique extensively used in systems such as soybean (*Glycine max*) and common bean (*Phaseolus vulgaris*) that have proven to obtain great benefits from this practice (Santos *et al*., 2019).

In general, developing a systematic breeding strategy that includes field-based experimentation needs to be done. Validation of parental performance, interactions among plant and strain genotypes, ability to nodulate effectively under stresses and the effects of established microbial populations on inoculum all need to be assessed under field conditions.

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9. APPENDICES

Appendix A

Table A. Yeast-Mannitol Broth and Yeast-Mannitol Agar media constituents (Vincent, 1970).

Constituents	
Mannitol	10.0 g
K_2HPO_4	0.5 g
$MgSO_4.7H_2O$	0.2 g
NaCl	0.1 g
Yeast Extract	0.5 g
Distilled water	1.0 L
For YMA	
YMB	1 L
Agar	15.0 g

Appendix B

Table B. Days to flower and days to maturity of 36 *Lens* species accessions under added Nitrogen, BASF 1435 Nodulator XL® (*Rlv*) and control treatments.

Accessions	**Days to flower**			**Days to maturity**		
	N	**R**	**C**	**N**	**R**	**C**
CDC Maxim	40	46.3	39.3	103.17	106.2	85.3
CDC Redberry	34	44.8	40.8	-	-	-
CDC Robin	33.5	39.3	38.8	-	-	-
CDC Milestone	33.5	35.3	35.5	-	-	-
CDC Asterix	35.8	45.5	45	-	-	-
CDC KR-1	45.5	49	44.5	99.17	103.7	88.3
CDC Greenstar	36.8	44.3	36	99.5	104.7	85.7
CDC QG-4	41.3	47.5	35.5	-	-	-
Eston	33	42	38	-	-	-
VIR-421	35	40.3	36	-	-	-
Lupa	34.3	38.8	38.3	93.5	98.33	93.7
ILL 7502	33.5	36.8	34.3	-	-	-
ILL 1704	31.3	33.3	31.8	-	-	-
ILL 8006	36.3	43	41	-	-	-
Indianhead	36.3	50	44.5	101.5	113.2	93.7
BGEO 16880	35.3	34.5	32.5	94.8	97	87.2
IG72529	31	33.3	34	-	-	-
IG72611	40.3	47.3	42.5	-	-	-
IG72622	59	51.3	48	-	-	-
IG72643	34	42.3	31.8	107.8	102.33	82.3
IG72672	31.5	34	33.5	-	-	-
PI 572376	40	35.8	42.5	107.7	119.7	88

Table B. Continued.

Accessions	**Days to flower**			**Days to maturity**		
	N	**R**	**C**	**N**	**R**	**C**
IG72613	32.5	33.3	37	-	-	-
IG72614	32.3	39.3	37.5	-	-	-
IG72805	33	33.5	35.8	-	-	-
PI 572390	34.3	33.5	32.5	106	110.5	101.33
IG72543	33.3	36.3	34.5	-	-	-
IG72623	41.8	47.5	49	115.33	118	92.7
IG 72760	42.3	42	47	101.83	103.7	88.3
IG 110810	36.3	44	35.8	106.7	112.3	97.2
IG 110813	36.5	39.5	39.8	-	-	-
IG 72537	35	43.8	36	-	-	-
IG 72815	48	51	43.8	-	-	-
L01-827A	33.5	38.3	43.5	97.8	116	85.7
LR59-81	32.5	38.5	35.3	111.8	118	113
IG 116024‡	-	-	-	-	-	-
Mean	38.6	41	36.6	103.3	108.8	91.6
LSD		4.81***			9.61***	

‡IG 116024 did not flower during the time of the experiment.

ANOVA values are ns: not significant, *, **, ***: significant at 0.05, 0.01, 0.001 respectively.

Appendix C

Table C. Analysis of variance (ANOVA), coefficient of variation (CV) and least significant difference (LSD) values for all evaluated parameters of 36 *Lens* species accessions under added N, BASF 1435 Nodulator XL® (*Rlv*) and control treatments on Chapter 3.

ANOVA	DTF	DTM	SDW	RDW	R:S	SPAD	NcontS	N ac
Plot (+N,R,C)	1.94***	6.33***	340.4***	63.8***	0.05**	1.69***	0.18***	4.26***
Block	0.94**	ns	ns	ns	ns	ns	0.10***	ns
Accession	2.78***	5.55***	556.9***	121.7***	0.11***	4.65***	0.30***	14.76***
Accession*Plot	4.82***	9.61***	964.5***	210.7***	0.2***	8.06***	0.52***	25.56***

ANOVA	N_2 fixed	NN	NDW	SpNDW	TRL	TRV	TRSA	RAD
Plot (+N,R,C)					1116.55***	1.36***	138.55***	0.29***
Block	9.84*	ns	ns	ns	ns	ns	ns	ns
Accession	29.52***	114.2***	29.72***	0.28***	1376.5***	2.12***	183.99***	0.42***
Accession*Plot					2384.17***	3.68***	318.68***	0.73***

ANOVA	SN	SW	KSW	HI	Protein
Plot (+N,R,C)	24.34***	0.41***	0.99***	1.66***	ns
Block	ns	ns	ns	ns	ns
Accession	46.92***	0.76***	2.56***	4.36***	0.52***
Accession*Plot	81.26***	1.31***	4.43***	7.55***	0.73***

ANOVA values are ns: not significant, *, **, ***: significant at 0.05, 0.01, 0.001 respectively. DTF: days to flower, DTM: days to maturity, SDW: shoot dry weight, RDW: root dry weight, R:S: root to shoot ratio, NcontS: N concentration in shoots, N ac: N accumulation per plant, NN: number of nodules, NDW: nodule dry weight, SpNDW: specific nodule dry weight, TRL: total root length, TRV: total root volume, TRSA: total root superficial area, RAD: root average diameter, SN: seed number, SW: seed weight, KSW: thousand seed weight, HI: harvest index and seed percentage protein.

Appendix D

Table D.1. Shoot dry weight (mg), root dry weight(mg) and Root:shoot ratio of 36 *Lens* species accessions under added Nitrogen, BASF 1435 Nodulator XL® (*Rlv*) and control treatments.

Accessions	Shoot dry weight (mg)			Root dry weight (mg)			Root:shoot ratio		
	N	R	C	N	R	C	N	R	C
CDC Maxim	2980.0	2370.0	1347.5	995.6	807.7	487.5	0.35	0.38	0.35
CDC Redberry	3455.0	2827.5	1082.5	958.6	506.6	433.9	0.27	0.18	0.44
CDC Robin	3515.0	3420.0	652.5	857.5	501.2	263.3	0.25	0.15	0.47
CDC Milestone	4250.0	2760.0	532.5	951.6	493.9	205.9	0.24	0.19	0.38
CDC Asterix	4475.0	3110.0	1087.5	820.0	440.3	435.2	0.19	0.14	0.40
CDC KR-1	3827.5	2930.0	687.5	1027.4	570.6	343.1	0.27	0.20	0.51
CDC Greenstar	3200.0	3837.5	1317.5	1094.3	623.8	276.3	0.35	0.16	0.23
CDC QG-4	4602.5	1892.5	420.0	1102.4	377.1	179.8	0.26	0.22	0.43
Eston	3595.0	1717.5	942.5	814.6	258.7	181.4	0.23	0.15	0.22
VIR-421	3787.5	1285.0	775.0	647.1	291.4	239.2	0.20	0.22	0.34
Lupa	3160.0	897.7	1227.5	695.7	335.8	321.2	0.23	0.34	0.27
ILL 7502	4707.5	1555.0	522.5	761.5	202.6	149.2	0.17	0.13	0.31
ILL 1704	2237.5	1037.5	570.0	507.9	160.1	130.8	0.24	0.16	0.22
ILL 8006	5485.0	1715.0	745.0	1104.9	220.6	274.4	0.20	0.14	0.38
Indianhead	4660.0	2840.0	1342.5	1087.9	567.6	427.4	0.23	0.19	0.35
BGEO 16880	2910.0	480.0	480.0	412.5	293.1	163.9	0.14	0.66	0.34
IG72529	3210.0	362.5	587.5	535.3	79.2	153.0	0.17	0.22	0.28
IG72611	3312.5	557.5	210.0	771.3	136.7	71.9	0.25	0.28	0.33
IG72622	3462.5	455.0	490.0	888.9	194.9	219.7	0.29	0.43	0.45
IG72643	4267.5	1127.5	902.5	706.6	88.5	237.7	0.17	0.10	0.38
IG72672	2855.0	432.5	727.5	517.4	61.3	174.9	0.18	0.16	0.27
PI 572376	3955.0	350.0	517.5	731.5	97.7	105.3	0.19	0.27	0.19
IG72613	3215.0	447.5	765.0	503.2	75.4	173.3	0.18	0.17	0.24
IG72614	2360.0	152.5	225.0	471.5	27.7	68.2	0.21	0.19	0.31

Table D.1. Continued.

IG72805	2547.5	472.5	367.5	508.4	86.6	153.3	0.20	0.29	0.43
PI 572390	2122.5	572.5	565.0	386.4	124.3	210.0	0.18	0.21	0.43
IG72543	2557.5	475.0	462.5	537.1	52.0	83.4	0.20	0.11	0.18
IG72623	3697.5	607.5	422.5	575.7	90.6	138.4	0.16	0.17	0.36
IG 72760	2905.0	1340.0	330.0	487.9	204.3	102.6	0.17	0.14	0.33
IG 110810	3085.0	397.5	267.5	342.5	148.8	81.9	0.12	0.36	0.32
IG 110813	2822.5	437.5	350.0	390.1	81.8	66.6	0.13	0.20	0.19
IG 72537	1882.5	310.0	292.5	347.1	41.9	71.6	0.18	0.15	0.24
IG 72815	1297.8	280.0	152.5	223.1	62.3	66.4	0.17	0.21	0.45
L01-827A	1967.5	147.5	355.0	272.1	18.2	128.3	0.14	0.14	0.34
LR59-81	2377.5	632.5	322.5	502.8	95.6	60.1	0.18	0.14	0.24
IG 116024	2205.0	460.0	240.0	741.1	157.6	100.2	0.35	0.34	0.41
Mean				0.676	0.238	0.194	0.21	0.22	0.33
LSD		0.96^{***}			0.21^{***}			0.2^{***}	

ANOVA values are ns: not significant, *, **, ***: significant at 0.05, 0.01, 0.001 respectively.

Table D.2. N concentration in shoots (%) and total N accumulation (mg/plant) at flowering of 36 *Lens* species accession with added Nitrogen, BASF 1435 Nodulator XL® (*Rlv*) and control treatments.

Accessions	N concentration (%)			Total N accumulation (mg/plant)		
	N	R	C	N	R	C
CDC Maxim	1.79	3.25	1.13	54.07	77.54	15.29
CDC Redberry	2.41	3.17	1.55	81.83	89.54	17.18
CDC Robin	1.69	2.55	1.32	59.43	86.30	9.14
CDC Milestone	1.98	2.95	1.16	88.35	82.55	6.23
CDC Asterix	2.46	3.23	1.42	112.77	101.86	15.24
CDC KR-1	2.42	3.47	1.36	89.79	102.37	9.23
CDC Greenstar	1.61	2.9	1.55	50.93	110.96	19.51
CDC QG-4	2.41	3.08	1.49	111.11	56.93	6.33
Eston	2.1	2.67	1.13	75.23	45.73	10.14
VIR-421	2.3	2.93	1.49	91.24	37.44	11.47
Lupa	1.52	1.19	1.1	45.18	13.14	9.55
ILL 7502	1.47	2.2	1.04	69.24	34.46	5.48
ILL 1704	2.42	2.58	1.25	52.51	26.31	6.80
ILL 8006	2.14	2.41	1.17	117.53	41.17	8.21
Indianhead	2.23	3.54	1.53	104.15	101.24	19.25
BGEO 16880	1.83	2.22	1.1	51.91	10.67	5.22
IG72529	1.8	3.18	1.9	54.66	11.67	10.20
IG72611	1.77	2.61	2.22	57.58	12.58	4.72
IG72622	2.03	3.16	1.97	73.09	14.40	9.86
IG72643	2.06	2.77	1.39	86.16	30.57	12.02
IG72672	1.96	2.88	1.59	53.47	12.43	11.87
PI 572376	1.56	3.26	1.54	61.46	11.27	7.81
IG72613	2.23	3.4	1.42	71.51	15.16	10.51
IG72614	2.61	2.53	1.42	62.41	3.91	3.28
IG72805	2.32	3.5	1.32	58.09	13.25	6.17
PI 572390	2.82	3.26	1.26	57.54	18.54	7.58
IG72543	2.67	2.5	1.19	63.73	11.96	5.65
IG72623	2.13	3.34	1.7	78.74	20.21	7.80
IG 72760	2.04	3.02	1.6	58.93	40.08	4.88
IG 110810	1.94	2.59	1.59	57.95	10.40	4.26
IG 110813	1.94	2.67	1.66	54.95	10.47	5.79
IG 72537	2.51	2.06	1.72	45.51	6.52	5.04
IG 72815	2.98	2.36	1.85	38.55	6.24	2.84
L01-827A	1.76	2.27	1.99	25.76	6.63	3.38
LR59-81	2.42	2.85	1.91	46.86	16.67	6.11

Table D-2. Continued.

IG 116024	2.1	3.86	2.3	47.57	17.72	5.36
Mean	2.12	2.84	1.51	66.94	36.17	8.78
LSD		0.52***			25.56***	

ANOVA values are ns: not significant, *, **, ***: significant at 0.05, 0.01, 0.001 respectively.

Appendix E

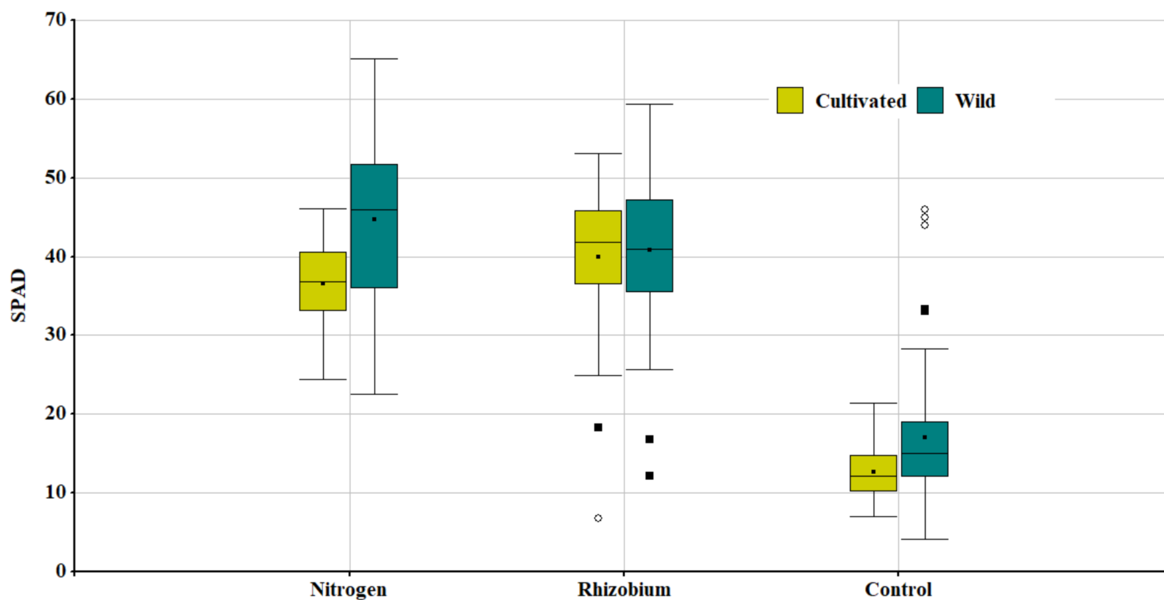

Figure E. Means of SPAD measurements at flowering of 36 *Lens* species accession with added Nitrogen, BASF 1435 Nodulator XL® (*Rlv*) and control treatments. (LSD=8.06***, P=0.001).

Appendix F

Table F. Total root length, total root volume total root superficial area and root average diameter of 36 *Lens* species accessions with added Nitrogen, BASF 1435 Nodulator XL® (*Rlv*) and control treatments.

Accessions	Total root length (cm)			Total root volume (cm^3)			Total root superficial area (cm^2)			Root average diameter (mm)		
	N	R	C	N	R	C	N	R	C	N	R	C
CDC Maxim	9847.0	8123.0	2444.1	15.2	15.3	6.5	1476.0	1212.6	340.5	2.5	2.3	1.7
CDC Redberry	8730.5	5876.4	5506.8	15.3	8.2	8.3	1311.7	778.0	755.3	3.4	1.6	1.8
CDC Robin	9746.5	7418.0	1719.7	17.3	11.5	4.8	1453.7	1032.4	374.5	3.4	1.9	0.9
CDC Milestone	12638.9	5983.9	2615.9	17.9	9.1	3.3	1681.8	827.2	327.2	3.1	1.8	0.9
CDC Asterix	9570.8	4909.2	3800.2	12.9	7.3	7.9	1216.7	666.4	607.3	1.9	1.2	1.4
CDC KR-1	11940.1	6432.6	3288.2	17.5	10.0	5.9	1615.6	895.5	471.9	2.5	1.8	1.4
CDC Greenstar	11056.1	6560.5	2925.8	16.9	9.7	2.5	1494.1	891.0	287.2	2.9	2.0	0.8
CDC QG-4	12106.5	4582.4	2841.5	18.4	6.0	4.3	1670.6	489.6	414.9	2.8	1.1	1.3
Eston	10946.8	2297.7	1698.4	17.1	2.2	3.1	1525.7	250.7	243.5	3.8	0.6	0.8
VIR-421	8118.2	4159.0	2541.2	11.2	5.3	4.1	1063.1	524.7	359.6	2.5	1.0	1.0
Lupa	7318.2	4187.9	2567.9	13.4	7.3	5.7	1102.6	621.7	426.4	2.7	1.4	1.2
ILL 7502	9480.2	2653.3	1566.3	13.8	3.3	2.1	1272.3	329.7	206.9	3.1	0.9	0.6
ILL 1704	6562.6	1465.4	1142.3	10.0	2.0	2.4	908.5	189.8	182.5	2.5	0.4	0.6
ILL 8006	10414.6	1865.2	2993.6	16.1	3.0	4.6	1667.7	263.8	430.8	2.2	0.5	1.1
Indianhead	15644.7	7785.1	3263.0	20.5	10.4	6.9	2021.2	1003.4	514.5	3.1	1.8	1.4
BGEO 16880	4881.2	1272.9	1653.4	6.4	1.5	2.6	623.5	155.1	232.4	1.4	0.5	0.6
IG72529	7249.1	1043.4	1545.8	10.7	1.4	3.0	984.1	133.9	240.6	1.8	0.4	1.0
IG72611	8577.7	1699.4	655.9	12.4	2.2	1.0	1152.0	215.9	88.8	2.1	0.5	0.4
IG72622	9224.4	2041.5	2411.8	12.1	2.7	4.0	1178.8	261.3	346.3	1.8	0.7	0.9
IG72643	7316.6	960.6	1176.8	11.6	1.3	2.1	1028.8	123.4	173.7	2.1	0.5	0.6
IG72672	5470.7	730.5	1818.9	8.7	0.8	2.6	769.1	83.6	243.8	2.0	0.3	0.6
PI 572376	9083.2	1506.3	2013.7	12.7	1.9	3.5	1201.0	187.8	293.6	1.8	0.6	0.9
IG72613	5594.2	1007.6	1647.9	9.8	1.4	3.2	819.4	133.9	256.8	1.7	0.4	0.8

Table F. Continued.

IG72614	5653.8	412.8	1011.9	7.7	0.5	1.1	821.5	48.8	114.7	2.2	0.4	0.4
IG72805	6105.0	1397.0	1934.3	10.3	1.8	3.1	885.9	175.0	273.3	2.1	0.5	0.9
PI 572390	4699.8	1571.7	1965.9	9.0	2.4	4.0	725.0	217.6	307.9	2.0	0.7	1.0
IG72543	3061.4	514.0	699.7	2.7	0.4	1.1	390.7	49.2	97.7	0.9	0.3	0.4
IG72623	7111.6	848.8	967.1	11.1	1.4	2.1	992.8	123.2	157.5	2.1	0.5	0.6
IG 72760	5650.0	1785.5	1052.7	8.1	3.5	1.6	751.0	276.4	144.4	1.3	0.8	0.4
IG 110810	4283.7	1732.1	574.5	5.4	2.6	1.5	533.9	236.1	121.8	1.1	0.7	0.5
IG 110813	4915.6	743.3	564.9	7.8	1.1	1.0	686.2	99.1	84.3	1.7	0.4	0.5
IG 72537	3656.2	784.2	608.8	7.0	1.1	1.2	564.7	105.6	96.4	1.7	0.5	0.5
IG 72815	3206.2	1063.0	994.8	3.9	1.2	1.5	395.7	127.8	134.0	0.8	0.5	0.4
L01-827A	3366.7	268.7	1360.1	4.5	0.3	2.4	434.8	28.8	200.7	1.0	0.3	0.8
LR59-81	6340.5	1349.5	691.4	8.8	1.4	0.9	836.2	153.0	87.5	2.1	0.5	0.4
IG 116024	9550.8	2640.4	1583.3	13.0	3.0	1.8	1245.3	312.2	190.2	2.0	0.7	0.5
Mean	7753.1	2768.7	1884.7	11.6	4.0	3.3	1069.3	367.3	273.0	2.2	0.9	0.8
LSD $_{(P \geq 0.05)}$		2384.17***			3.68***			318.68***			0.73***	

ANOVA values are ns: not significant, *, **, ***: significant at 0.05, 0.01, 0.001 respectively.

Appendix G

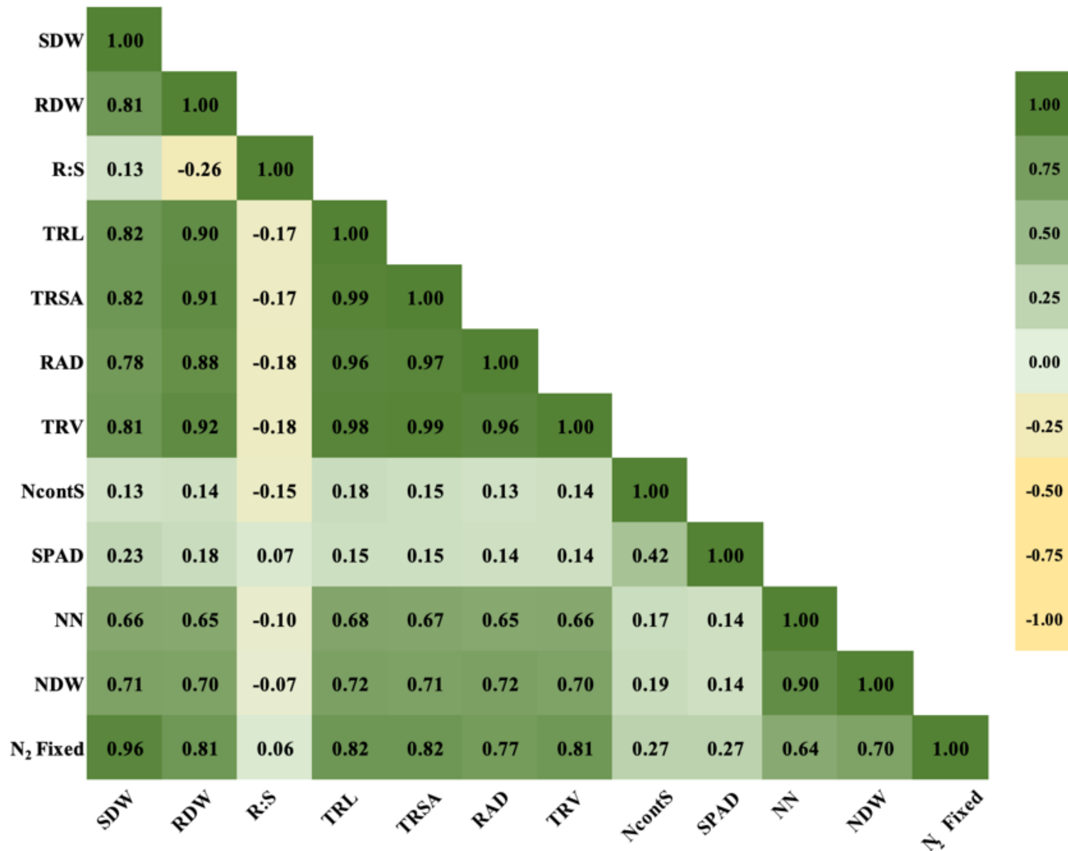

Figure G. Pearson Correlation among parameters evaluated at flowering of 36 *Lens* species accessions inoculated with BASF 1435 Nodulator XL® (*Rlv*). SDW: *s*hoot dry weight, RDW: root dry weight, R:S: root to shoot ratio, TRL: total root length, TRSA: total root superficial area, RAD: root average diameter, TRV: total root volume, NcontS: N concentration in shoots, SPAD: leaf chlorophyll concentration, NN: number of nodules, NDW: nodule dry weight and N_2 fixed.

Appendix H

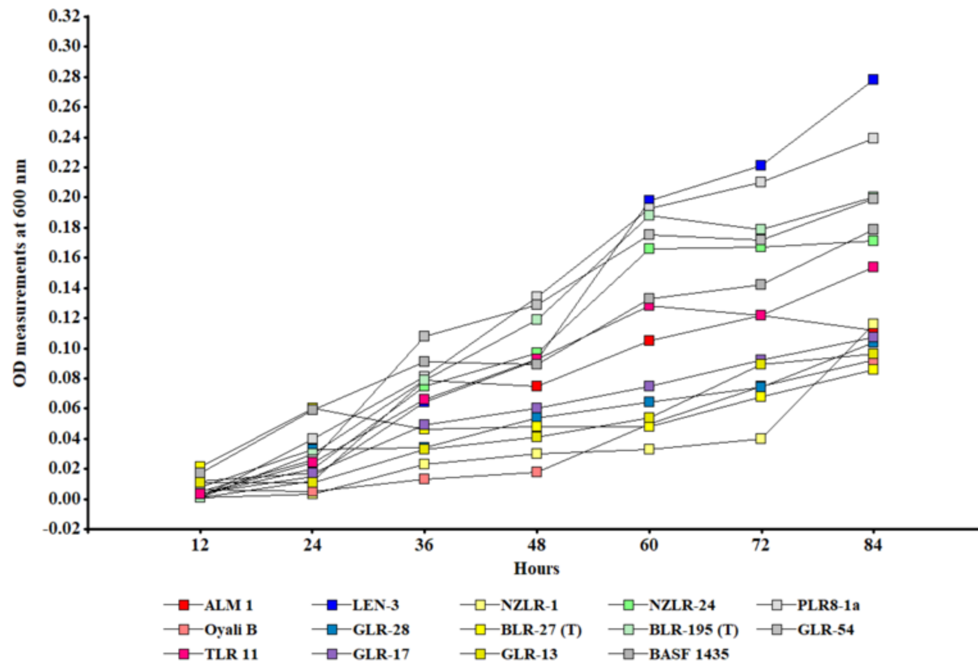

Figure H. Strain growth optical density at 600 nm of 14 *Rhizobium* strains at 12, 24, 36, 48, 60, 72 and 84 hours on yeast mannitol broth shaked at 180 rpm.

Appendix I

Table I.1. Characteristics of the 0- to 30 cm soil layer of 3 site-years in Saskatchewan, Canada, 2017-2018.

Property	**Site-year§**		
	Sutherland 17§	**Sutherland 18**	**Clavet 18**
Soil texture	clay loam	loam	loam
pH (1:2 water)	7.6	7.2	7.4
Organic matter	3.6	3.4	3.2
NO_3-N, mg kg^{-1}	7	3	35
P, mg kg^{-1}	27	21	30
K, mg kg^{-1}	>270	>300	>250
Electric conductivity (1:2 water)	0.3	0.1	0.2

§Soil records of Rosthern 2018 were not available.

Table I.2. Agronomic management during the crop cycle in all 4 site-years.

	Application time	Primary Target
Edge® MicroActiv (ethalfluralin)	Fall	broadleaf and grassy weeds
Glyfosate	prior to emergence	broadleaf and grassy weeds
Pursuit® 240 (imazethapyr)	Early post-emergent	broadleafs
Centurion® (clethodim)	post-emergent	grasses
Heat® (saflufenacil)	pre-harvest	pre-harvest desiccation

Appendix J

Table J.1. Shoot dry weight and N concentration in shoots of CDC Maxim by site-year with 15 *Rhizobium* treatments, 2017-2018.

Strain	**Shoot Dry Weight (g)**				**Nitrogen concentration in shoots (%)**			
	Sutherland 17	**Rosthern 18**	**Sutherland 18**	**Clavet 18**	**Sutherland 17**	**Rosthern 18**	**Sutherland 18**	**Clavet 18**
ALM 1	2.1	3.56	4.18	2.17	2.84	2.58	2.89	3.06
BLR-27 (T)	1.56	3.41	4.41	1.72	2.80	2.58	2.75	3.08
BLR-195 (T)	1.51	3.6	4.27	2.05	2.71	2.48	2.74	2.92
GLR-13	1.67	3.18	4.33	2.16	2.75	2.53	2.83	2.87
GLR-17	1.89	3.51	4.57	1.77	2.73	2.65	2.68	2.87
GLR-28	1.72	2.85	4.26	1.78	2.73	2.63	2.73	2.83
GLR-54	1.97	3.16	4.24	2.6	2.72	2.58	2.89	2.7
LEN-3	1.92	2.97	4.53	1.93	2.87	2.7	2.63	3.25
NZLR-1	1.92	2.36	4.58	2.21	2.86	2.64	2.73	3.22
NZLR-24	1.78	3.54	4.55	1.91	2.91	2.62	2.65	3.45
Oyali B	1.81	2.99	3.81	2.26	2.74	2.65	2.89	2.66
PLR8-1a	1.54	3.3	4.08	2.05	2.81	2.65	2.77	3.02
TLR 11	2.07	2.77	4.66	2.31	2.75	2.55	2.68	3.02
BASF1435	2.13	2.73	4.8	2	2.80	2.62	2.66	3.11
Non-inoculated	1.68	3.29	4.58	2.13	2.67	2.61	2.95	2.46
LSD $_{(\geq 0.05)}$		NS				0.21		

NS: not significant, LSD: least significant difference value with a P≥0.05.

Table J.2. Total number of nodules, number of nodules from 0-15 cm, from 15-30 cm and nodule colour of CDC Maxim by site-year with 15 *Rhizobium* treatments, 2017-2018.

Strain	Total number of nodules				Number of nodules (0-15 cm)			
	Sutherland 17	Rosthern 18	Sutherland 18	Clavet 18	Sutherland 17	Rosthern 18	Sutherland 18	Clavet 18
ALM 1	29.5	58.8	66.0	59.8	19.5	44.3	51.8	43.0
BLR-27 (T)	32.0	42.8	75.8	52.3	22.3	34.8	59.0	40.0
BLR-195 (T)	30.0	36.8	77.3	59.8	19.3	29.3	60.5	45.8
GLR-13	33.0	37.0	78.0	76.5	21.5	30.3	59.8	57.8
GLR-17	29.8	37.8	73.8	45.0	21.5	29.5	58.0	33.8
GLR-28	30.8	33.8	80.8	45.3	18.8	27.5	59.8	35.0
GLR-54	31.5	35.3	65.3	57.0	21.5	29.0	50.3	44.0
LEN-3	31.0	33.8	80.5	58.8	22.5	27.8	61.3	40.0
NZLR-1	28.8	33.5	68.8	56.5	18.5	27.3	53.5	42.3
NZLR-24	27.5	44.8	69.8	35.3	18.8	35.8	53.8	29.8
Oyali B	27.5	37.5	66.3	54.0	18.5	30.3	49.3	41.8
PLR8-1a	22.5	34.8	74.5	61.3	14.3	27.3	58.0	46.8
TLR 11	26.0	35.0	72.0	61.0	17.3	29.8	55.0	47.5
BASF1435	21.8	35.0	71.5	49.0	14.3	29.3	53.5	38.8
Non-inoculated	27.5	36.8	74.5	67.5	18.3	30.0	57.5	50.3
LSD $_{(\geq 0.05)}$		ns				ns		

NS: not significant, LSD: least significant difference value with a P≥0.05.

Table J.2. Continued.

Strain	Number of nodules (15-30 cm)				Nodule colour			
	Sutherland 17	Rosthern 18	Sutherland 18	Clavet 18	Sutherland 17	Rosthern 18	Sutherland 18	Clavet 18
ALM 1	10.0	14.8	14.5	16.8	2.2	3.0	2.9	2.5
BLR-27 (T)	9.8	8.0	17.0	12.8	2.6	2.8	2.9	2.4
BLR-195 (T)	10.5	7.5	16.8	15.5	2.6	2.5	2.9	2.4
GLR-13	11.5	7.0	18.5	18.5	2.6	2.9	3.0	2.5
GLR-17	8.3	8.0	14.8	10.8	2.6	2.7	2.9	2.3
GLR-28	12.0	6.3	20.8	10.5	2.3	2.7	2.9	2.0
GLR-54	10.0	6.0	14.8	13.3	2.4	2.9	3.0	2.7
LEN-3	8.5	6.3	19.0	18.5	2.6	2.9	3.0	2.5
NZLR-1	10.0	6.3	15.5	14.0	2.4	2.3	2.9	2.2
NZLR-24	8.3	8.5	16.3	5.5	2.5	3.0	3.1	2.2
Oyali B	8.8	7.0	17.3	12.5	2.4	2.8	3.0	2.3
PLR8-1a	8.3	7.5	16.3	14.5	2.4	2.7	2.9	2.4
TLR 11	8.8	5.5	17.0	13.0	2.2	2.6	3.0	2.4
Non-inoculated	8.8	6.5	17.0	17.3	2.2	2.8	2.6	2.3
LSD $_{(\geq 0.05)}$	ns				0.21			

NS: not significant, LSD: least significant difference value with a P≥0.05.

Table J.3. Thousand seed mass (g), seed percentage protein and yield (kg ha-1) of CDC Maxim by site-year with 15 Rhizobium treatments, 2017-2018.

Strain	**Thousand seed mass (g)**				**Seed percentage protein**			
	Sutherland 17	**Rosthern 18**	**Sutherland 18**	**Clavet 18**	**Sutherland 17**	**Rosthern 18**	**Sutherland 18**	**Clavet 18**
ALM 1	54.8	43.0	43.2	42.0	25.8	23.4	25.1	24.3
BLR-27 (T)	56.2	43.8	41.1	39.8	25.6	23.9	25.1	24.9
BLR-195 (T)	54.1	43.7	42.5	39.9	25.6	23.4	30.8	24.3
GLR-13	53.2	42.0	43.3	39.0	25.4	23.8	27.4	24.8
GLR-17	60.4	43.6	40.6	36.7	25.5	23.6	25.1	25.0
GLR-28	54.7	38.5	42.6	39.9	25.8	23.7	25.0	24.9
GLR-54	49.7	44.6	43.4	42.6	25.5	23.8	24.9	24.6
LEN-3	57.5	40.3	43.1	42.1	25.6	23.8	24.9	24.8
NZLR-1	53.3	41.3	42.1	42.7	24.5	23.6	24.9	24.6
NZLR-24	47.5	42.9	40.7	42.0	25.2	23.8	25.0	25.2
Oyali B	54.3	43.3	40.7	40.4	25.4	23.2	25.2	24.8
PLR8-1a	57.3	42.4	41.0	40.1	25.3	23.7	24.9	24.3
TLR 11	49.2	43.8	42.1	38.2	25.6	23.6	25.3	24.7
BASF1435	47.8	44.0	42.1	38.9	25.5	23.6	24.7	24.7
Non-inoculated	54.3	42.1	40.6	41.1	25.9	23.5	24.9	24.7
LSD $_{(\geq 0.05)}$		2.6				1.91		

NS: not significant, LSD: least significant difference value with a P≥0.05.

Table J.3. Continued.

Strain	Yield (kg ha^{-1})			
	Sutherland 17	**Rosthern 18**	**Sutherland 18**	**Clavet 18**
ALM 1	2284.7	2153.6	2513.7	3268.8
BLR-27 (T)	2215.3	2216.4	2528.5	3360.8
BLR-195 (T)	2124.7	2037.3	2354.3	3423.0
GLR-13	2255.5	2114.0	2529.4	3388.2
GLR-17	2156.3	2281.9	2422.8	3358.9
GLR-28	2152.0	1984.8	2504.3	3234.8
GLR-54	2342.6	1960.4	2481.7	3387.5
LEN-3	2314.5	2533.3	2533.2	3718.5
NZLR-1	2417.5	2349.0	2508.1	3278.5
NZLR-24	2632.7	2546.3	2595.8	3801.4
Oyali B	2221.5	2200.9	2445.3	3445.0
PLR8-1a	2341.2	2174.4	2555.9	3793.0
TLR 11	2282.4	2179.0	2480.3	3256.9
BASF1435	2334.2	2257.3	2223.4	3634.8
Non-inoculated	2298.5	2037.7	2321.7	3373.7
LSD $_{(\geq 0.05)}$	165.3			

NS: not significant, LSD: least significant difference value with a P≥0.05.

Appendix K

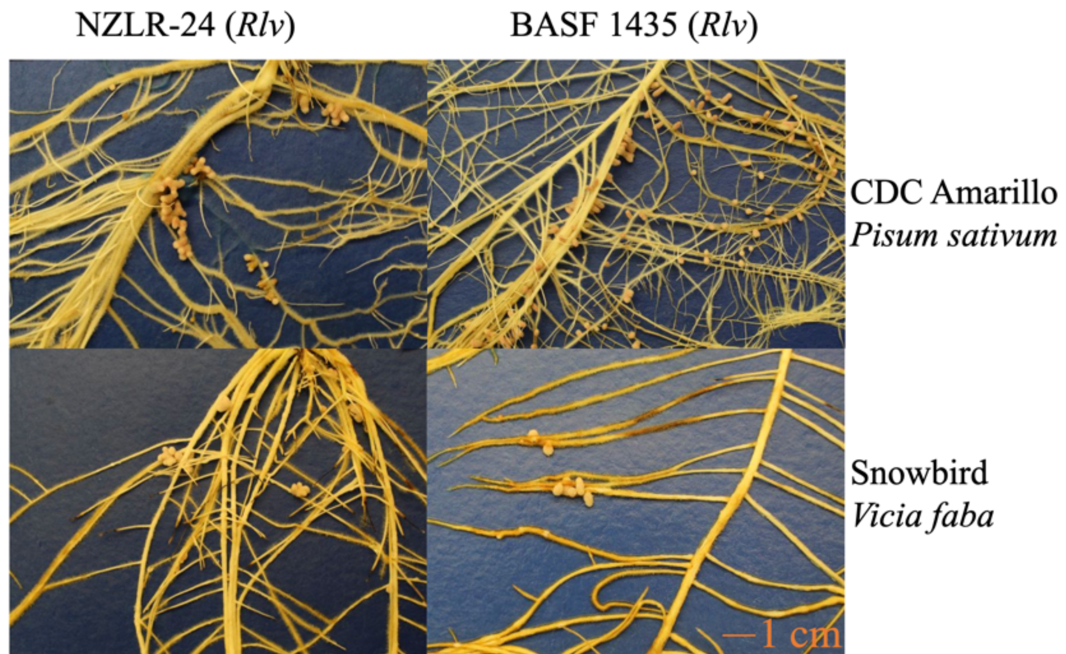

Figure K. Nodulation in CDC Amarillo (Top) and Snowbird (bottom), with the strains NZLR-24 (left) and BASF 1435 (right) in growing pouches 30 days after inoculation.

Appendix L

Table L. Analysis of variance (ANOVA), coefficient of variation (CV) and least significant difference (LSD) values for all evaluated parameters on Chapter 4.

ANOVA	DTF	DTM	SDW	TNN	NN1	NN2	NC	NcontS	KSM	Protein	Yield
Block	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Siteyear	ns	1.7^{**}	0.21^{***}	5.63^{***}	4.42^{***}	1.76^{***}	0.18^{***}	0.19^{***}	1.1^{***}	0.84^{***}	250.9^{***}
Strain	ns	ns	ns	ns	ns	ns	0.17^{*}	0.11^{***}	ns	ns	124.31^{***}
Siteyear*Strain	ns	ns	ns	ns	ns	ns	0.21^{*}	0.14^{***}	2.6^{***}	1.91^{**}	165.32^{***}
CV	55.2	44.2	17.84	32.3	31.2	43.56	11.57	6.25	5.91	7.82	9.72

ANOVA values are ns: not significant, *, **, ***: significant at 0.05, 0.01, 0.001 respectively. SDW: shoot dry weight, TNN: total number of nodules, NN1: NN 0-15 cm, NN2: NN 15-30 cm, NC: nodule colour, NcontS: N concentration in shoots, KSW: thousand seed mass and Protein: seed percentage protein.

Appendix M

Table M. N-free Nutrient Solution utilized to fertilize plants of experiments from Chapter 5 (Broughton and Dilworth, 1970).

Stock Solution	Chemical	g/liter
1	CaCl2•2H2O	294.1
2	KH2PO4	136.1
3	$FeC_6H_5O_7 \cdot 3H_2O$	6.7
	$MgSO_4 \cdot 7H_2O$	123.3
	K_2SO_4	87.0
	$MnSO_4 \cdot H_2O$	0.338
4	H_3BO_3	0.247
	$ZnSO_4 \cdot 7H_2O$	0.288
	$CuSO_4 \cdot 5H_2O$	0.100
	$CoSO_4 \cdot 7H_2O$	0.056
	$NaMoO_2 \cdot 2H_2O$	0.048

Appendix N

Table N. Nodule fresh weight (mg) of 10 *Lens* species accessions inoculated with one of 5 *Rhizobium* strains treatments.

Accessions	**BLR-27 (T)**	**PLR8-1a**	**NZLR-24**	**Oyali B**	**BASF 1435**
	NFW	**NFW**	**NFW**	**NFW**	**NFW**
CDC Greenstar	548.4	524.3	1191.8	422.3	359.1
Eston	620.5	706.9	506.1	589.5	417.8
Lupa	149.8	154.8	349.5	93.5	91.3
VIR-421	555.8	625.8	448.1	144.1	194.3
BGE 016880	170.0	22.6	293.3	171.3	353.8
IG 72643	121.3	139.3	166.7	333.2	141.6
IG 72623	292.0	78.6	441.0	119.6	110.7
PI 572390	181.9	28.0	367.4	448.0	286.5
IG 110810	75.1	17.0	270.5	241.6	211.5
L01-827A	12.7	10.3	93.6	66.0	106.9
Mean	272.8	230.8	412.8	262.9	227.4
LSD $_{(\geq 0.05)}$			32.16^{***}		

ANOVA values are ns: not significant, *, **, ***: significant at 0.05, 0.01, 0.001 respectively.

Appendix O

Table O. Analysis of variance (ANOVA), coefficient of variation (CV) and least significant difference (LSD) values for all evaluated parameters of 10 *Lens* accessions inoculated with one of five *Rhizobium* strains or +N treatments evaluated in Chapter 5.

ANOVA	**SDW40**	**NcontS**	**SNY**	**RDW40**	**NcontR**	**RNY**	**R:S**	**N_2Fixed**	**NN**	**NDW**	**SpNDW**
T (Strains, +N)	0.65***	0.009***	15.56***	0.05***	0.038***	1.14***	0.031***	16.33***	2.94***	6.2**	ns
Block	0.46**	0.006***	11.04*	0.034***	0.027***	0.803***	0.021**	11.54**	ns	ns	ns
Accession	0.15***	0.08***	4.67***	0.04***	0.013***	1.03***	0.03***	5.27***	1.74***	4.9***	0.29***
Accession*T	0.37***	0.19***	11.44***	0.1***	0.033***	2.53***	0.07***	12.92***	3.9***	10.85***	0.65***
CV	9.17	7.14	12.41	15.91	1.7	13.1	29.79	13.26	3.03	30.55	120.99

ANOVA	**Efficiency**	**SN**	**SW**	**KSW**	**Protein**	**PY**	**HI**	**DTF**	**DTM**	**NFW**
T (Strains, +N)	15.85***	58.09***	0.89***	2.51***	0.03***	0.2***	0.02***	1.36**	1.77***	7.71***
Block	ns	ns	ns	ns	ns	ns	ns	ns	ns	5.97***
Accession	19.96***	35.92***	1.18***	2.97***	0.22***	0.24***	0.03***	1.63***	1.67***	160.82***
Accession*T	37.93***	87.98***	2.89***	7.3***	0.53***	0.59***	0.07***	4.00***	4.09***	32.16***
CV	24.62	35.17	45.36	25.01	2.13	43.65	13.85	5.74	2.47	99.72

ANOVA values are ns: not significant, *, **, ***: significant at 0.05, 0.01, 0.001 respectively. SDW: shoot dry weight, NcontS: N concentration in shoots, SNY: shoot N yield, RDW: root dry weight, NcontR: N concentration in roots, RNY: root N yield, R:S: root to shoot ratio, N_2 fixed, NN: number of nodules, NDW: nodule dry weight, SpNDW: specific nodule dry weight, Efficiency: efficiency of nodulation, SN: seed number, SW: seed weight, KSW: thousand seed weight, Protein: seed percentage protein, PY: seed protein yield, HI: harvest index, DTF: days to flower and DTM: days to maturity.

Appendix P

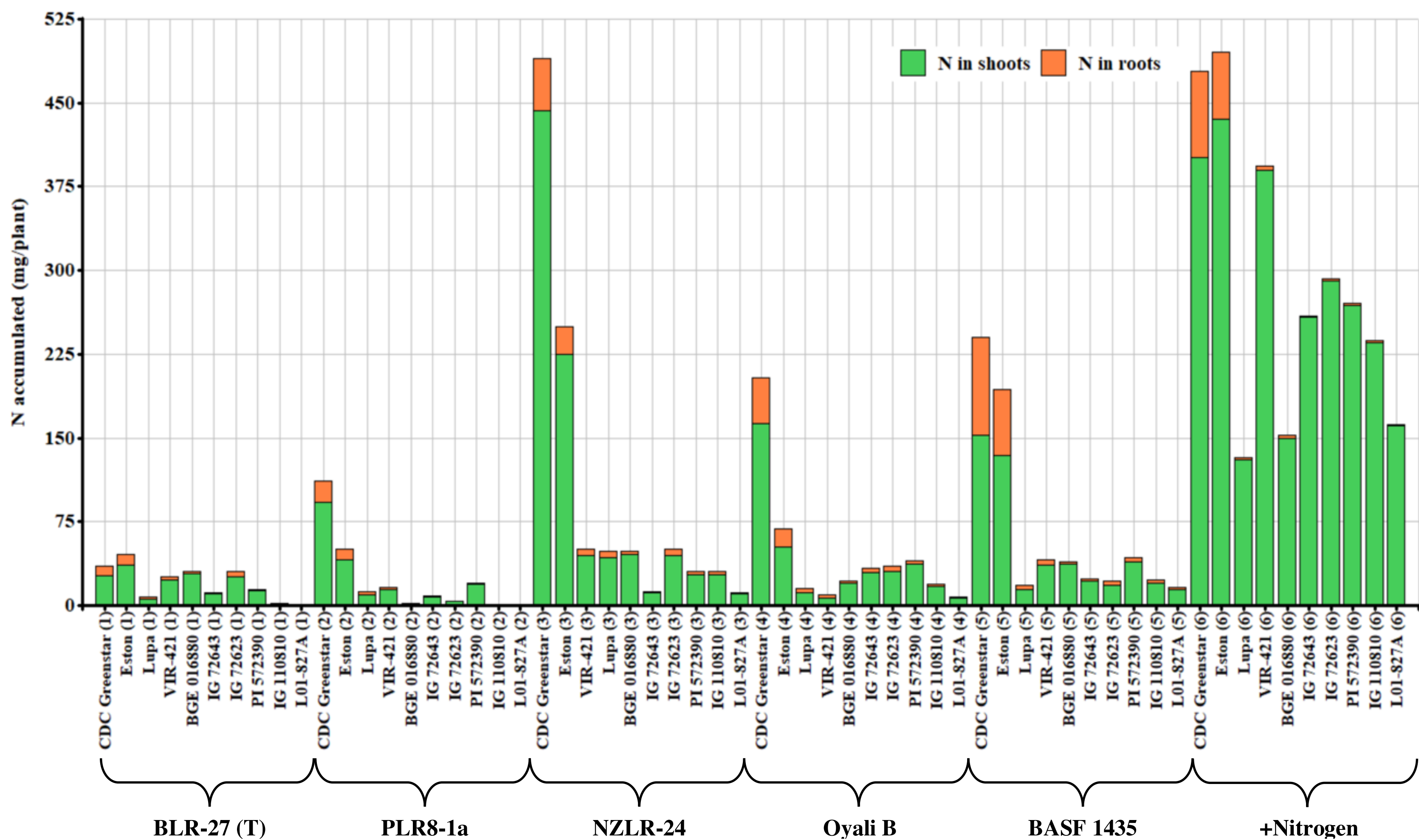

Figure P. Proportion of N accumulated in shoots and roots of 10 *Lens* species accessions inoculated with one of five *Rhizobium* strains or added N treatments.

Appendix Q

Table Q. Nitrogen concentration in shoots and roots mg g^{-1} of 10 *Lens* accessions inoculated with one of 5 *Rhizobium* strains or added N treatments.

Accessions	**BLR-27 (T)**		**PLR8-1a**		**NZLR-24**		**Oyali B**		**BASF 1435**		**Nitrogen**	
	Nshoot	**Nroot**	**Nshoot**	**Nroot**	**Nshoot**	**Nroot**	**Nshoot**	**Nroot**	**Nshoot**	**Nroot**	**Nshoot**	**Nroot**
CDC Greenstar	2.2	2.6	2.6	1.8	3.3	2.2	2.1	1.8	2.4	1.6	2.6	1.7
Eston	1.9	2.7	2.4	1.5	2.3	1.4	1.9	1.6	2.4	1.6	2.8	1.7
Lupa	2.0	1.9	2.5	1.8	2.1	1.8	2.1	1.4	1.9	1.3	1.7	0.8
VIR-421	3.0	2.3	3.0	1.8	2.1	1.8	2.2	2.5	2.6	1.9	2.5	1.7
BGE 016880	2.5	1.8	2.4	1.6	1.9	1.6	1.6	1.4	1.7	1.1	1.2	1.4
IG 72643	2.9	2.0	2.3	1.5	3.4	1.8	2.1	2.2	2.2	1.9	2.2	1.3
IG 72623	3.1	2.1	2.2	1.5	2.8	1.9	2.0	1.8	2.6	2.0	2.6	1.2
PI 572390	3.0	1.9	2.2	1.4	2.4	1.6	2.1	1.8	2.2	1.3	2.1	1.4
IG 110810	1.9	1.8	2.3	1.4	2.6	1.8	1.9	2.2	2.3	1.7	1.8	1.5
L01-827A	1.3	1.8	2.4	1.2	3.3	0.8	2.1	1.8	1.9	1.7	1.4	1.3
Mean	2.4	2.1	2.4	1.5	2.6	1.7	2.0	1.8	2.2	1.6	2.1	1.4
LSD $_{(\geq 0.05)}$	N concentration in shoots= 0.19***, N concentration in roots= 0.033***											

ANOVA values are ns: not significant, *, **, ***: significant at 0.05, 0.01, 0.001 respectively.

Appendix R

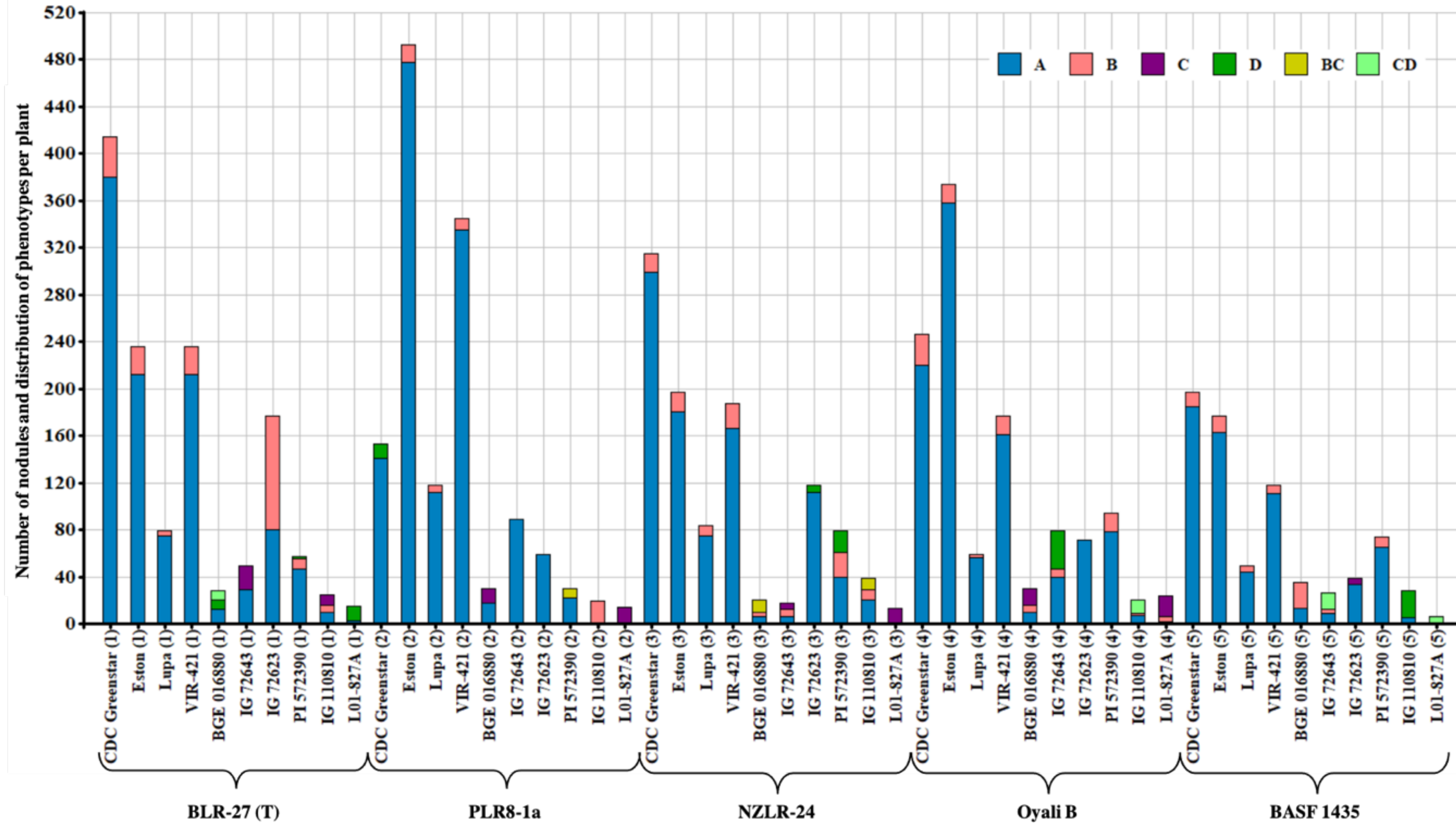

Figure R. Number of nodules and distribution of nodule phenotypes per plant of 10 *Lens* species accessions inoculated with one of five *Rhizobium* strains from 3 species at 40 days after seeding. A: determinate, and indeterminate B: bifurcate, C: palmate and D: coralloid.

Appendix S

Table S.1. Seed number, seed weight (g) and thousand seed weight (g) of 10 *Lens* species accessions inoculated with one of 5 *Rhizobium* strains or added N treatments.

Accessions	**BLR-27 (T)**			**PLR8-1a**			**NZLR-24**		
	SN	**SW**	**KSW**	**SN**	**SW**	**KSW**	**SN**	**SW**	**KSW**
CDC Greenstar	174.0	11.7	67.5	34.0	2.2	63.9	495.0	24.3	49.4
Eston	253.0	9.3	37.0	129.0	4.7	38.6	441.0	17.4	38.2
Lupa	41.0	1.7	41.2	9.0	0.3	36.6	126.0	3.8	35.4
VIR-421	99.0	2.4	24.5	21.0	0.4	22.3	313.0	8.4	25.2
BGE 016880	72.0	1.2	16.9	3.0	0.0	12.7	149.0	2.7	17.6
IG 72643	141.0	1.8	12.5	6.0	0.1	11.7	305.0	7.0	20.1
IG 72623	362.0	5.8	16.1	20.0	0.3	12.2	408.0	6.4	15.6
PI 572390	95.0	1.5	34.0	11.0	0.1	9.7	314.0	7.8	20.4
IG 110810	11.0	0.1	9.6	0.0	0.0	nd	245.0	4.9	23.3
L01-827A	1.0	0.0	30.0	2.0	0.0	2.3	200.0	1.9	9.0
Mean	124.7	3.6	28.8	23.5	0.8	23.3	299.4	8.5	25.4
LSD	seed number= 88***, seed weight= 2.89***, thousand seed weight=7.3***								

ANOVA values are ns: not significant, *, **, ***: significant at 0.05, 0.01, 0.001 respectively. nd: not defined.

Table S.1. Continued.

Accessions	Oyali B			BASF 1435			Nitrogen		
	SN	**SW**	**KSW**	**SN**	**SW**	**KSW**	**SN**	**SW**	**KSW**
CDC Greenstar	316.0	18.0	57.1	342.0	20.3	59.3	407.0	24.0	58.9
Eston	134.0	5.1	36.8	437.0	16.7	38.3	466.0	17.1	36.7
Lupa	12.0	0.5	38.9	40.0	1.7	44.0	195.0	6.8	34.9
VIR-421	108.0	0.9	16.2	247.0	6.5	26.3	250.0	6.2	24.7
BGE 016880	126.0	2.2	17.5	156.0	2.7	17.4	292.0	4.5	15.5
IG 72643	384.0	7.9	20.8	329.0	4.5	13.7	556.0	7.0	19.7
IG 72623	226.0	3.5	15.1	221.0	3.4	15.3	615.0	8.5	13.9
PI 572390	264.0	3.6	13.4	241.0	3.1	13.0	458.0	6.3	13.7
IG 110810	211.0	3.9	18.6	267.0	4.7	17.7	356.0	5.4	15.4
L01-827A	69.0	0.4	6.4	345.0	2.5	7.3	667.0	5.7	9.6
Mean	184.9	4.6	24.1	262.2	6.6	25.2	426.3	9.5	24.3
LSD	seed number= 88***, seed weight= 2.89***, thousand seed weight=7.3***								

ANOVA values are ns: not significant, *, **, ***: significant at 0.05, 0.01, 0.001 respectively. nd: not defined.

Table S.2. Seed percentage protein, protein yield (mg/plant) and harvest index (%) of 10 *Lens* species accessions inoculated with one of 5 *Rhizobium* strains or added N treatments.

Accessions	**BLR-27 (T)**			**PLR8-1a**			**NZLR-24**			**Oyali B**			**BASF 1435**			**Nitrogen**		
	P	**PY**	**HI**	**P**	**PY**	**HI**	**P**	**PY**	**HI**	**P**	**PY**	**HI**	**P**	**PY**	**HI**	**P**	**PY**	**HI**
CDC Greenstar	18.3	2.1	56	18.5	0.4	48	19.8	4.7	52	17.3	3.1	49	18.3	3.7	54	18.6	4.5	54
Eston	19.4	1.8	52	20.4	1.0	48	22.3	3.9	55	19.9	1.0	50	21.4	3.6	57	21.5	3.7	58
Lupa	23.9	0.4	58	16.5	0.1	45	22.1	0.8	53	20.7	0.1	53	18.0	0.4	57	18.4	1.2	58
VIR-421	22.4	0.5	49	19.1	0.1	49	22.9	2.0	54	21.7	0.2	51	25.5	1.7	56	24.1	1.5	53
BGE 016880	24.2	0.3	49	nd	0.1	22	24.4	0.7	43	22.2	0.5	47	23.6	0.6	42	20.6	0.9	45
IG 72643	24.8	0.4	47	24.3	0.0	38	22.7	1.6	48	22.9	1.8	52	27.2	1.2	54	22.1	2.4	41
IG 72623	24.1	1.4	51	18.3	0.1	32	23.1	1.5	49	22.9	0.8	46	24.5	0.8	47	24.1	2.1	47
PI 572390	21.8	0.3	55	15.0	0.0	44	22.9	1.6	56	22.7	0.8	47	26.7	0.8	54	21.8	1.4	51
IG 110810	18.9	0.0	25	nd	<0.01	0	25.0	1.3	39	26.2	1.0	41	27.1	1.3	44	25.6	1.4	45
L01-827A	20.9	<0.01	25	nd	<0.01	7	22.4	0.5	45	21.7	0.1	46	23.9	0.6	47	21.9	1.2	50
Mean	21.80	0.8	47	18.9	0.2	33	22.8	1.9	49	21.8	1.0	48	23.6	1.5	51	21.9	2.0	50
LSD	seed percentage protein= 0.53***, protein yield= 0.59***, harvest index= 7***.																	

ANOVA values are ns: not significant, *, **, ***: significant at 0.05, 0.01, 0.001 respectively. nd: not defined.

Table S.3. Shoot dry weight (g), root dry weight (g) and root to shoot ratio of 10 *Lens* species accessions inoculated with one of 5 *Rhizobium* strains or added N treatments.

Accession	**BLR-27 (T)**			**PLR8-1a**			**NZLR-24**		
	SDW	**RDW**	**R:S**	**SDW**	**RDW**	**R:S**	**SDW**	**RDW**	**R:S**
CDC Greenstar	1.2	0.4	0.31	3.5	1.0	0.29	13.4	2.2	0.17
Eston	1.9	0.4	0.19	1.7	0.6	0.37	9.6	1.8	0.19
Lupa	0.3	0.1	0.43	0.4	0.2	0.44	2.0	0.3	0.18
VIR-421	0.8	0.2	0.21	0.5	0.1	0.24	2.0	0.3	0.18
BGE 016880	1.1	0.1	0.11	0.1	0.0	0.22	2.4	0.2	0.09
IG 72643	0.4	0.1	0.12	0.3	0.1	0.18	0.4	0.0	0.10
IG 72623	0.8	0.2	0.28	0.2	0.1	0.36	1.6	0.3	0.19
PI 572390	0.4	0.1	0.17	0.8	0.1	0.16	1.2	0.2	0.18
IG 110810	0.1	0.0	0.37	0.0	0.0	0.77	1.1	0.2	0.19
L01-827A	0.0	0.0	0.23	0.0	0.0	0.22	0.3	0.0	0.10
Mean	0.7	0.2	0.24	0.8	0.2	0.33	3.4	0.6	0.16

Accession	**Oyali B**			**BASF 1435**			**Nitrogen**		
	SDW	**RDW**	**R:S**	**SDW**	**RDW**	**R:S**	**SDW**	**RDW**	**R:S**
CDC Greenstar	7.6	2.2	0.30	6.4	5.6	0.87	15.3	4.4	0.29
Eston	2.8	1.8	0.36	5.7	3.8	0.66	15.4	3.4	0.22
Lupa	0.6	0.3	0.49	0.8	0.3	0.34	7.5	0.2	0.03
VIR-421	0.3	0.3	0.34	1.4	0.3	0.19	15.4	0.2	0.01
BGE 016880	1.3	0.2	0.12	2.1	0.2	0.11	12.6	0.2	0.01
IG 72643	1.4	0.0	0.13	1.0	0.1	0.13	12.0	0.1	0.01
IG 72623	1.5	0.3	0.21	0.7	0.2	0.24	11.0	0.1	0.01
PI 572390	1.8	0.2	0.10	1.8	0.2	0.13	12.8	0.2	0.01
IG 110810	0.9	0.2	0.13	0.9	0.2	0.19	13.2	0.1	0.01
L01-827A	0.3	0.0	0.11	0.8	0.1	0.12	11.5	0.1	0.01
Mean	1.8	0.6	0.23	2.2	1.1	0.3	12.7	0.9	0.06
LSD	shoot dry weight= 0.37***, root dry weight= 0.1***, root to shoot ratio= 0.07***.								

ANOVA values are ns: not significant, *, **, ***: significant at 0.05, 0.01, 0.001 respectively.

Table S.4. Days to flowering and days to maturity of 10 *Lens* accessions inoculated with one of 5 *Rhizobium* strains or added N treatments.

Accession	**BLR-27 (T)**		**PLR8-1a**		**NZLR-24**		**Oyali B**		**BASF 1435**		**Nitrogen**	
	DTF	**DTM**	**DTF**	**DTM**	**DTF**	**DTM**	**DTF**	**DTM**	**DTF**	**DTM**	**DTF**	**DTM**
CDC Greenstar	48.7	112.0	52.0	105.0	47.7	107.3	46.3	110.7	47.0	110.0	50.0	110.0
Eston	39.3	100.3	38.3	94.3	38.7	99.0	41.0	91.7	40.7	102.0	43.3	101.0
Lupa	44.0	101.3	50.0	95.3	46.7	106.7	50.0	102.0	45.3	97.7	45.3	97.3
VIR-421	37.3	110.3	38.0	106.3	39.3	119.7	42.3	107.0	40.3	126.7	39.7	121.0
BGE 016880	35.0	96.3	38.7	94.0	34.7	94.0	36.0	95.0	36.3	102.3	40.0	98.0
IG 72643	47.3	98.0	50.0	95.0	46.7	105.0	48.0	109.0	48.0	98.3	47.3	97.0
IG 72623	45.3	99.0	51.0	95.7	48.3	108.0	51.0	96.0	50.3	107.7	50.0	107.0
PI 572390	35.7	93.0	37.7	93.3	37.0	99.7	36.0	99.0	38.0	99.3	37.3	103.7
IG 110810	55.0	91.3	42.0	89.7	45.7	105.0	46.7	103.3	49.0	105.3	42.3	106.3
L01-827A	29.0	102.0	41.7	92.3	38.7	109.0	38.7	105.0	40.0	107.7	39.0	106.0
Mean	41.7	100.4	43.9	96.1	42.3	105.3	43.6	101.9	43.5	105.7	43.4	104.7
LSD $_{(\geq 0.05)}$	DTF= 4.0***, DTM= 4.09***											

ANOVA values are ns: not significant, *, **, ***: significant at 0.05, 0.01, 0.001 respectively.

Appendix T

Table T.1. List of RILs of the LR-68 population (IG 72643 *L. orientalis* × CDC Greenstar *L. culinaris*) evaluated for N fixation related traits inoculated with the strain NZLR-24 (*Rhizobium leguminosarum* bv. *viciae*), grown in a cabinet in plastic pouches and evaluated 30 DAS.

Entry	Name	Entry	Name	Entry	Name	Entry	Name
1	IG 72643	33	LR68-76	65	LR68-132	97	LR68-181
2	CDC Greenstar	34	LR68-77	66	LR68-135 ▸	98	LR68-182
3	LR68-4	35	LR68-79	67	LR68-137	99	LR68-183
4	LR68-5	36	LR68-80	68	LR68-139	100	LR68-184
5	LR68-6	37	LR68-81	69	LR68-140	101	LR68-186
6	LR68-7	38	LR68-83	70	LR68-143	102	LR68-187
7	LR68-8	39	LR68-85	71	LR68-144	103	LR68-188
8	LR68-9	40	LR68-86	72	LR68-148	104	LR68-189
9	LR68-12	41	LR68-87	73	LR68-149	105	LR68-190
10	LR68-19	42	LR68-88	74	LR68-150	106	LR68-193
11	LR68-22	43	LR68-89	75	LR68-151	107	LR68-194
12	LR68-25 ▸	44	LR68-91	76	LR68-152	108	LR68-201
13	LR68-26	45	LR68-93	77	LR68-154	109	LR68-203
14	LR68-30	46	LR68-95	78	LR68-155	110	LR68-204
15	LR68-33	47	LR68-96	79	LR68-157	111	LR68-205
16	LR68-34	48	LR68-100	80	LR68-158	112	LR68-208
17	LR68-35	49	LR68-102	81	LR68-159	113	LR68-216
18	LR68-43	50	LR68-104	82	LR68-161	114	LR68-217
19	LR68-45	51	LR68-105	83	LR68-162	115	LR68-218
20	LR68-49	52	LR68-107	84	LR68-163	116	LR68-219
21	LR68-50	53	LR68-108	85	LR68-165	117	LR68-221
22	LR68-57	54	LR68-109	86	LR68-166	118	LR68-222
23	LR68-58	55	LR68-112	87	LR68-167	119	LR68-223
24	LR68-59	56	LR68-113 ▸	88	LR68-168	120	LR68-226
25	LR68-62	57	LR68-114	89	LR68-169	121	LR68-227
26	LR68-63	58	LR68-116	90	LR68-170	122	LR68-228
27	LR68-64	59	LR68-117	91	LR68-171	123	LR68-229
28	LR68-66	60	LR68-119	92	LR68-172		
29	LR68-67	61	LR68-124	93	LR68-173		
30	LR68-68	62	LR68-125	94	LR68-175		
31	LR68-72	63	LR68-129	95	LR68-176		
32	LR68-75	64	LR68-131	96	LR68-180		

▸ not included in the QTL mapping.

Table T.2. List of RILs of the LR-70 population (Eston *L. culinaris* × IG 72623 *L. odemensis*) evaluated for N fixation related traits inoculated with the strain NZLR-24 (*Rhizobium leguminosarum* bv. *viciae*), grown in a cabinet in plastic pouches and evaluated 30 DAS.

Entry	Name	Entry	Name	Entry	Name	Entry	Name
1	IG 72623	33	LR70-59 ‣	65	LR70-132	97	LR70-179
2	Eston	34	LR70-60	66	LR70-133	98	LR70-180 ‣
3	LR70-2	35	LR70-61	67	LR70-134	99	LR70-181
4	LR70-3	36	LR70-63	68	LR70-135	100	LR70-183
5	LR70-5	37	LR70-71	69	LR70-136	101	LR70-184
6	LR70-6	38	LR70-73	70	LR70-137	102	LR70-188
7	LR70-8	39	LR70-75	71	LR70-138	103	LR70-189
8	LR70-10	40	LR70-76	72	LR70-139	104	LR70-190
9	LR70-13	41	LR70-82	73	LR70-140	105	LR70-192
10	LR70-14	42	LR70-83	74	LR70-142	106	LR70-194
11	LR70-15	43	LR70-84	75	LR70-143	107	LR70-196
12	LR70-16	44	LR70-85	76	LR70-144	108	LR70-199
13	LR70-17	45	LR70-87	77	LR70-146	109	LR70-200
14	LR70-18	46	LR70-88	78	LR70-149	110	LR70-205
15	LR70-20	47	LR70-90	79	LR70-150	111	LR70-206
16	LR70-22	48	LR70-94	80	LR70-151	112	LR70-207
17	LR70-24	49	LR70-96	81	LR70-154	113	LR70-208
18	LR70-27	50	LR70-97	82	LR70-157	114	LR70-209
19	LR70-29	51	LR70-99	83	LR70-158	115	LR70-210
20	LR70-35	52	LR70-105	84	LR70-159	116	LR70-211
21	LR70-36 ‣	53	LR70-110	85	LR70-160	117	LR70-212
22	LR70-38	54	LR70-111	86	LR70-162	118	LR70-213
23	LR70-39	55	LR70-115	87	LR70-163 ‣	119	LR70-214
24	LR70-42	56	LR70-116	88	LR70-165	120	LR70-215
25	LR70-43	57	LR70-120	89	LR70-166	121	LR70-216 ‣
26	LR70-45	58	LR70-121	90	LR70-167		
27	LR70-47	59	LR70-122	91	LR70-168 ‣		
28	LR70-48	60	LR70-123	92	LR70-170		
29	LR70-50	61	LR70-126	93	LR70-173 ‣		
30	LR70-53	62	LR70-127	94	LR70-174		
31	LR70-57	63	LR70-130	95	LR70-175		
32	LR70-58	64	LR70-131	96	LR70-177 ‣		

‣ not included in the QTL mapping

Table T.3. List of RILs of the LR-86 population (Lupa *L. culinaris* × BGE 016880 *L. orientalis*) evaluated for N fixation related traits inoculated with the strain NZLR-24 (*Rhizobium leguminosarum* bv. *viciae*), grown in a cabinet in plastic pouches and evaluated 30 DAS.

Entry	Name	Entry	Name	Entry	Name
1	Lupa7	33	LR-86-39	65	LR-86-73
2	BGE 016880	34	LR-86-40	66	LR-86-74
3	LR-86-1	35	LR-86-41	67	LR-86-75
4	LR-86-2	36	LR-86-42	68	LR-86-77
5	LR-86-3	37	LR-86-43	69	LR-86-80
6	LR-86-4	38	LR-86-44	70	LR-86-81
7	LR-86-5	39	LR-86-45	71	LR-86-84
8	LR-86-6	40	LR-86-46	72	LR-86-85
9	LR-86-9	41	LR-86-47	73	LR-86-87
10	LR-86-10	42	LR-86-48	74	LR-86-88
11	LR-86-11	43	LR-86-49	75	LR-86-90
12	LR-86-12	44	LR-86-50	76	LR-86-91
13	LR-86-13	45	LR-86-52	77	LR-86-92
14	LR-86-14	46	LR-86-54	78	LR-86-93
15	LR-86-15	47	LR-86-55	79	LR-86-96
16	LR-86-16	48	LR-86-56	80	LR-86-97
17	LR-86-17	49	LR-86-57	81	LR-86-98 ‣
18	LR-86-18	50	LR-86-58	82	LR-86-99
19	LR-86-19	51	LR-86-59	83	LR-86-100 ‣
20	LR-86-21	52	LR-86-60	84	LR-86-101
21	LR-86-23	53	LR-86-61 ‣	85	LR-86-102
22	LR-86-24	54	LR-86-62	86	LR-86-103
23	LR-86-25	55	LR-86-63	87	LR-86-104
24	LR-86-27	56	LR-86-64	88	LR-86-105
25	LR-86-28	57	LR-86-65	89	LR-86-106
26	LR-86-29	58	LR-86-66	90	LR-86-107
27	LR-86-31	59	LR-86-67	91	LR-86-108
28	LR-86-32	60	LR-86-68	92	LR-86-109
29	LR-86-34	61	LR-86-69	93	LR-86-110 ‣
30	LR-86-35	62	LR-86-70	94	LR-86-111 ‣
31	LR-86-36	63	LR-86-71	95	LR-86-112 ‣
32	LR-86-37	64	LR-86-72		

‣ not included in the QTL mapping

Appendix U

Table U. Estimates of variance components and broad sense heritability of N fixation related traits observed in RILs of the LR-68, LR-70 and LR-86 populations inoculated with the strain NZLR-24 (*Rhizobium leguminosarum* bv. *viciae*), grown in a cabinet in plastic pouches and evaluated 30 DAS.

Population	Variance component	SFW	SDW	RFW	RDW	NFW	NDW	NN	SpNFW	SpNDW
LR-68	σ^2G	0.0006	0.0006	0.04	0.0002	643.5	5.9	47.3	1.92	0.005
	σ^2e	0.0021	0.0021	0.09	0.0004	2582.7	21.4	114.6	10.08	0.018
	σ^2P	0.0027	0.0027	0.13	0.0006	3226.2	27.3	161.9	11.99	0.023
	H^2 (broad sense)	0.22	0.22	0.30	0.43	0.2	0.22	0.29	0.16	0.20
	Variance component	**SFW**	**SDW**	**RFW**	**RDW**	**NFW**	**NDW**	**NN**	**SpNFW**	**SpNDW**
LR-70	σ^2G	0.032	0.0012	0.07	0.0002	3070.46	57.56	959.9	0.15	0.005
	σ^2e	0.021	0.0008	0.04	0.0002	3043.05	61.0	607.8	0.28	0.015
	σ^2P	0.053	0.002	0.11	0.0004	6113.51	118.6	1567.7	0.43	0.019
	H^2 (broad sense)	0.60	0.60	0.63	0.50	0.50	0.49	0.61	0.34	0.24
	Variance component	**SFW**	**SDW**	**RFW**	**RDW**	**NFW**	**NDW**	**NN**	**SpNFW**	**SpNDW**
LR-86	σ^2G	0.093	0.004	0.24	0.0007	2778.4	73.3	186.4	0.78	0.030
	σ^2e	0.065	0.003	0.17	0.0005	3970.4	96.5	148.8	4.51	0.123
	σ^2P	0.160	0.007	0.41	0.0012	6748.7	169.8	335.2	5.29	0.150
	H^2 (broad sense)	0.59	0.58	0.59	0.58	0.41	0.43	0.56	0.15	0.20

σ^2e: environmental contribution to total phenotypic variation, σ^2G: genetic variance, σ^2P: total phenotypic variation, H^2: broad sense heritability.

Appendix V

Table V. F-test results of ANOVA of N fixation related traits observed in RILs of the LR-68, LR-70 and LR-86 interspecific populations inoculated with the strain NZLR-24 (*Rhizobium leguminosarum* bv. *viciae*), grown in a cabinet in plastic pouches and evaluated 30 DAS.

Population		**Phenotypic traits**								
LR-68		**SFW**	**SDW**	**RFW**	**RDW**	**NFW**	**NDW**	**NN**	**SpNFW**	**SpNDW**
Effect	**df**	**F values**								
Line	120	2.14^{***}	1.79^{***}	2.3^{***}	2.22^{***}	1.75^{*}	1.82^{***}	2.23^{***}	1.57^{**}	1.77^{***}
Replication	2	56.92^{***}	33.54^{***}	69.8^{***}	79.09^{***}	21.91^{***}	63.03^{***}	76.51^{***}	2.16^{ns}	9.14^{***}
CV%		56.4	62.8	68.8	38.7	48.1	45.3	50.6	48.9	45.6
LR-70		**SFW**	**SDW**	**RFW**	**RDW**	**NFW**	**NDW**	**NN**	**SpNFW**	**SpNDW**
Effect	**df**	**F values**								
Line	118	5.53^{***}	5.4^{***}	6.0^{***}	4.57^{***}	4.03^{***}	3.83^{***}	5.74^{***}	2.57^{***}	1.92^{***}
Replication	2	21.55^{***}	12.17^{***}	20.93^{***}	23.4^{***}	6.43^{**}	5.4^{**}	1.29^{ns}	9.1^{***}	11.73^{***}
CV%		45.9	46.3	31.5	30.4	46.6	40.1	50.1	32.6	48.5
LR-86		**SFW**	**SDW**	**RFW**	**RDW**	**NFW**	**NDW**	**NN**	**SpNFW**	**SpNDW**
Effect	**df**	**F values**								
Line	92	6.69^{***}	6.41^{***}	6.67^{***}	6.73^{***}	3.8^{***}	4.04^{***}	6.01^{***}	1.69^{***}	2.0^{***}
Replication	3	5.51^{**}	6.03^{***}	3.91^{**}	4.07^{**}	4.86^{**}	6.68^{***}	1.69^{ns}	1.15^{ns}	2.66^{ns}
CV%		40.3	40.6	35.4	29.9	46.3	53.9	43.1	52.4	44.4

ANOVA values are ns: not significant, *, **, ***: significant at 0.05, 0.01, 0.001 respectively. df: degrees of freedom, CV: coefficient of variation. SFW: shoot fresh weight, SDW: shoot dry weight, RFW: root fresh weight, RDW: root dry weight, NFW: nodule fresh weight, NDW: nodule dry weight, NN: number of nodules, SpNFW: specific nodule fresh weight, SpNDW: specific nodule dry weight.

Appendix W

Table W. Means (± standard error), range and heritability of N fixation related traits of RIL from the interspecific populations LR-68 (IG 72643 *L. orientalis* × CDC Greenstar *L. culinaris*), LR-70 (Eston *L. culinaris* × IG 72623 *L. odemensis*) and LR-86 (Lupa *L. culinaris* × BGE 016880 *L. orientalis*) inoculated with the strain NZLR-24 (*Rhizobium leguminosarum* bv. *viciae*), grown in a cabinet in plastic pouches and evaluated 30 DAS.

Population	Trait	Mean±SE	Range	H^{2a}
LR-68	SFW	0.37±0.14	0.01-2.02	0.22
IG 72643 × CDC Greenstar	RFW	0.89±0.27	0.02-4.57	0.30
	NFW	53.3±29.3	0.3-580	0.20
	SDW	0.08±0.03	0.009-0.53	0.22
	RDW	0.06±0.01	0.003-0.2	0.33
	NDW	6.9±2.67	0.05-36.3	0.22
	SpNFW	2.43±1.83	0.2-41.4	0.16
	SpNDW	0.29±0.08	0.02-1.4	0.20
	NN	23.08±6.18	0.2-124.0	0.45
LR-70	SFW	0.32±0.08	0.01-2.1	0.60
(Eston × IG 72623)	RFW	0.65±0.11	0.01-2.9	0.63
	NFW	82.42±31.85	0-632.5	0.50
	SDW	0.06±0.02	0.003-0.39	0.60
	RDW	0.04±0.008	0.003-0.1	0.50
	NDW	12.05±4.51	0-90.2	0.49
	SpNFW	1.62±0.31	0-7.3	0.34
	SpNDW	0.25±0.07	0-1.9	0.24
	NN	49.53±14.23	0-220.0	0.61
LR-86	SFW	0.63±0.03	0.11-2.6	0.59
Lupa × BGE 016880	RFW	1.16±0.2	0.05-5.2	0.59
	NFW	112.4±31.5	12.5-596.4	0.41
	SDW	0.13±0.03	0.03-0.5	0.58
	RDW	0.08±0.01	0.002-0.28	0.58
	NDW	15.5±4.9	0.96-111.9	0.43
	SpNFW	4.04±1.06	0.96-31.3	0.15
	SpNDW	0.54±0.18	0.09-6.2	0.20
	NN	28.7±6.1	4-130.0	0.56

SFW: shoot fresh weight (g), SDW: shoot dry weight (g), RFW: root fresh weight (g), RDW: root dry weight (g), DFW: nodule fresh weight (mg), NDW: nodule dry weight (mg), NN: number of nodules, SpNFW: specific nodule fresh weight (mg), SpNDW: specific nodule dry weight (mg). [a]Broad sense heritability.

Appendix X

Table X. Phenotypic correlations among N fixation related traits of RIL from the interspecific populations LR-68 (IG 72643 *L. orientalis* × CDC Greenstar *L. culinaris*), LR-70 (Eston *L. culinaris* × IG 72623 *L. odemensis*) and LR-86 (Lupa *L. culinaris* × BGE 016880 *L. orientalis*) inoculated with the strain NZLR-24 (*Rhizobium leguminosarum* bv. *viciae*), grown in a cabinet in plastic pouches and evaluated 30 DAS.

Populations	Traits[a]	SFW	RFW	NFW	SDW	RDW	NDW	SpNFW	SpNDW	NN
LR-68	SFW	1								
	RFW	0.89^{***}	1							
	NFW	0.56^{***}	0.49^{***}	1						
	SDW	0.88^{***}	0.77^{**}	0.49^{***}	1					
	RDW	0.91^{***}	0.95^{**}	0.52^{***}	0.77^{***}	1				
	NDW	0.84^{***}	0.77^{***}	0.59^{***}	0.85^{***}	0.75^{***}	1			
	SpNFW	0.12^{*}	0.07^{ns}	0.80^{***}	0.10^{*}	0.13^{**}	0.08^{ns}	1		
	SpNDW	0.44^{***}	0.41^{***}	0.30^{***}	0.49^{***}	0.44^{***}	0.52^{***}	0.28^{***}	1	
	NN	0.68^{***}	0.65^{***}	0.50^{***}	0.62^{***}	0.60^{***}	0.79^{***}	-0.05^{ns}	0.03^{*}	1
LR-70	SFW	1								
	RFW	0.92^{***}	1							
	NFW	0.85^{***}	0.79^{***}	1						
	SDW	0.98^{***}	0.91^{***}	0.84^{***}	1					
	RDW	0.83^{***}	0.93^{***}	0.59^{***}	0.85^{***}	1				
	NDW	0.78^{***}	0.73^{***}	0.92^{***}	0.77^{***}	0.48^{***}	1			
	SpNFW	0.24^{ns}	0.26^{***}	0.37^{***}	0.23^{***}	0.19^{***}	0.25^{***}	1		
	SpNDW	0.01^{ns}	0.01^{ns}	0.07^{ns}	0.01^{ns}	-0.10^{*}	0.22^{***}	0.55^{***}	1	
	NN	0.78^{***}	0.73^{***}	0.90^{***}	0.77^{***}	0.55^{***}	0.87^{***}	0.09^{ns}	-0.05^{ns}	1
LR-86	SFW	1								
	RFW	0.92^{***}	1							
	NFW	0.82^{***}	0.82^{***}	1						
	SDW	0.99^{***}	0.90^{***}	0.81^{***}	1					
	RDW	0.91^{***}	0.97^{***}	0.76^{***}	0.90^{***}	1				
	NDW	0.82^{***}	0.82^{***}	0.85^{***}	0.81^{***}	0.78^{***}	1			
	SpNFW	0.30^{***}	0.25^{***}	0.48^{***}	0.32^{***}	0.27^{***}	0.25^{***}	1		
	SpNDW	0.37^{***}	0.33^{***}	0.28^{***}	0.40^{***}	0.37^{***}	0.60^{***}	0.45^{***}	1	
	NN	0.66^{***}	0.69^{***}	0.77^{***}	0.62^{***}	0.61^{***}	0.72^{***}	-0.09^{ns}	0.02^{ns}	1

[a]SFW: shoot fresh weight, RFW: root fresh weight, NFW: nodule fresh weight, SDW: shoot dry weight, RDW: root dry weight, NDW: nodule dry weight, SpNFW: specific nodule fresh weight, SpNDW: Specific nodule dry weight, NN: number of nodules

Appendix Y

Table Y. Summary of QTLs identified for N fixation related traits of RILs from the interspecific populations LR-68 (IG 72643 *L. orientalis* × CDC Greenstar *L. culinaris*), LR-70 (Eston *L. culinaris* × IG 72623 *L. odemensis*) and LR-86 (Lupa *L. culinaris* × BGE 016880 *L. orientalis*) grown in a chamber in plastic pouches and inoculated with the strain NZLR-24 (*Rlv*).

Population	QTL	Trait+	LG†	Position (cM)	Left marker	Right marker	Confidence interval	Peak LOD	○%PVE	Add‡
LR-68	*qSFW-1*	SFW	1	40.4	1p172202065	1p233212270	33.42-51.32	3.1	4.61	0.04
	qSFW-2	SFW	2	285.4	2p406138960	2p407532450	265.5-290.3	4.4	8.34	0.05
	qSFW-3	SFW	3	47.4	3p235929984	3p239179204	36.5-56.2	5.9	11.11	0.06
	qSDW-1-1	SDW	1	40.4	1p172202065	1p233212270	33.42-69.32	5.2	6.44	0.01
	qSDW-1-2	SDW	1	115.5	1p536677953	1p531934591	106.14-135.2	3.6	4.28	0.01
	qSDW-3	SDW	3	47.4	3p235929984	3p239179204	36.5-56.2	7.8	12.6	0.02
	qSDW-4	SDW	4	49.9	4p55587488	4p55778135	29.6-51.3	3.9	6.36	0.01
	qRDW-2	RDW	2	285.4	2p406138960	2p407532450	265.5-290.3	3.4	8.63	0.01
	qRDW-3	RDW	3	47.4	3p235929984	3p239179204	36.5-56.2	4.2	11.1	0.01
	qRDW-7	RDW	7	51.9	7p31178036	7p32792357	46.7-61.4	3.8	9.51	0.01
LR-70	*qSFW-5*	SFW	5	50.8	5p286068972	5p304588612	45.8-54.3	9.8	6.9	0.13
	qSFW-6	SFW	6	44.5	6p278028293	6p285753019	31.5-47.5	16.1	9.6	0.15
	qSDW-5	SDW	5	50.9	5p304588612	5p323896208	42.4-52.4	8.8	16.1	0.02
	qSDW-6-1	SDW	6	45.1	6p278028293	6p285753019	41.2-52.3	4.2	5.3	0.01
	qRFW-2	RFW	2	65.9	2p546210601	2p566864366	55.5-68.3	23.0	24.4	0.28
	qRFW-5	RFW	5	52.2	5p402737731	5p412468557	45.3-53.2	10.3	9.9	0.16
	qRFW-6	RFW	6	17.0	6p15185878	6p15185885	10.5-32.5	4.4	3.1	0.08
	qRDW-2	RDW	2	59.5	2p393966359	2p403618447	52.5-72.3	4.4	10.2	0.006
	qRDW-5	RDW	5	52.1	5p402737731	5p412468557	45.3-53.1	8.4	28.9	0.009
	qRDW-6	RDW	6	17.0	6p15185878	6p15185885	10.5-31.5	6.1	14.5	0.006
LR-86	*qSFW-3*	SFW	3	928.1	3p408832758	3p408832764	920.2-935.4	4.0	16.0	-0.27
	qSFW-4	SFW	4	87.8	4p25789506	4p26691244	79.4-89.6	3.6	17.6	-0.14

Table Y. Continued.

qSDW-1	SDW	1	310.5	1p394661452	1p395094319	301.2-318.9	3.0	14.7	0.03
qSDW-3	SDW	3	928.1	3p408832758	3p408832764	920.2-935.4	3.9	16.7	-0.05
qSDW-4	SDW	4	87.8	4p25789506	4p26691244	79.4-89.6	3.3	16.7	-0.028
qRFW-1	RFW	1	316.0	1p394723828	1p392846828	308.1-328.3	4.1	6.5	0.24
qRFW-4-1	RFW	4	62.5	4p16831524	4p15776900	52.5-67.4	6.1	4.5	0.31
qRFW-4-2	RFW	4	87.8	4p25789506	4p26691244	79.4-89.6	11.7	8.3	-0.43
qRFW-6	RFW	6	342.3	6p239525416	6p235883012	338.5-355.5	3.4	9.2	0.22
qRDW-1	RDW	1	310.6	1p394661452	1p395094319	299.9-319.5	4.5	8.5	0.01
qRDW-4	RDW	4	160.2	4p46667701	4p50744349	154.2-162.3	3.3	16.0	-0.01

[°]%PVE: phenotypic variation explained by the marker. [‡]Add: estimated additive effect of the marker. [†]LG: linkage group. [+]NFW: nodule fresh weight, NDW: nodule dry weight, SpNFW: specific nodule fresh weigth, SpNDW, specific nodule dry weight, NN: number of nodules.