Assessing the Competitiveness of *Rhizobium* Strains Isolated from Manitoban Agricultural Soil on *Phaseolus vulgaris*

By

Mubashshir M. Bari

A Thesis Submitted to the Faculty of Graduate and Postdoctoral Studies of
The University of Manitoba
In partial Fulfillment of the Requirements of the Degree of

MSc.

Department of Microbiology
University of Manitoba
Winnipeg, Manitoba, Canada

Abstract

Legumes typically obtain much of their nitrogen through a symbiotic relationship with rhizobia that can fix atmospheric nitrogen and supply it to the plant. While most agricultural legumes have a commercially available *Rhizobium* inoculant that is either applied to seeds or added during planting, an inoculant for dry beans (*Phaseolus vulgaris*) is not readily available. To address the lack of a viable inoculant for dry beans, a collection of 168 rhizobia capable of nodulating navy beans was previously assembled from across Manitoba. From this collection, 15 strains representing the top 90th percentile were chosen based on greenhouse trials to identify those with superior symbiotic nitrogen-fixation traits. In this study, we developed and refined a multi-iteration, competition-based screening method to identify the most competitive strains for nodule occupancy. Equal numbers of each of the 15 selected strains were combined into a consortium and used to inoculate bean seedlings. Three weeks after inoculation, 20 crown nodules were pooled, surface-sterilized, and the rhizobia were isolated for use in inoculating new plant sets. This process was repeated over three consecutive iterations. In the final round, rhizobia from 20 crown nodules per replicate were isolated, a single colony from each nodule was purified, and the whole genome was sequenced. To determine which of the original strains persisted through the selection process, average nucleotide identity (ANI) analysis was used. Across six independent competition experiments involving 120 crown nodules, an isolate of *Rhizobium sophoriradicis* (UMR 279) and an isolate of *Rhizobium croatiense* (UMR 297) showed the highest cumulative frequency, with UMR 297 appearing in 5 out of 6 replicates and occupying 41% of all nodules tested, while UMR 279 also appeared in 5 out of 6 replicates and accounted for 31% of all nodules tested. These strains were further characterized and are strong candidates for potential development as inoculants for dry beans.

Acknowledgements

I would like to sincerely thank my supervisor, Dr. Ivan Oresnik, for his support and guidance throughout my graduate studies. He has been an incredible mentor and a steady source of support during both the exciting and overwhelming moments. I am especially grateful for the way he gave feedback, always honest and helpful, but never discouraging. Thank you for believing in me and helping me grow; I truly would not have made it this far without you.

I would also like to thank the past and present members of the Oresnik lab for their support and for helping me build confidence in myself. A special thank you to Anna and Ishani for always being willing to help and for patiently answering my many questions.

To my study group Rebecca P., Kylah, Teassa and Fran, thank you for being such an important part of this journey. The long days we spent studying and writing together made everything feel more manageable and a lot less lonely. I am also very grateful to Jenny, Tiffany, Anna S., Elaine, Fabian, and Juliana for their friendship and for being there through both the good and the more difficult times. A special thank you to Ushashi, whose support meant so much to me in ways both big and small, from encouragement to simply making me feel at home.

I would like to thank my co-supervisor, Dr. George diCenzo, and my committee members, Dr. Aleeza Gerstein and Dr. Marike Palmer, for their time and guidance. I also appreciate the members of the BENEFIT Journal Club for their helpful discussions and feedback.

Finally, I would like to thank the Department of Microbiology, as well as the past and present staff of the Microbiology General Office (shoutout to Steph, Cheryl, Aymsley, and Jacylyn!) I also thank the Microbiology Graduate Students' Association for helping build a supportive community.

Dedication

I would like to dedicate this work to my parents, Sonali and Mukta. Thank you for always being my support system and for standing by me through everything. You have been my constant source of strength, and I hope to make you proud.

I also dedicate this work to someone very special, my younger self. To the young queer kid who struggled with fear, self-doubt and was afraid to dream big: this is for you. I hope you are proud of who you have become.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ALA – 5-aminiolevulinic acid

ANI – Average nucleotide identity

BNF – Biological nitrogen fixation

CCRH – Curled, colonized root hair

CI – Competitive Index

DMSO – Dimethyl sulfoxide

DPI – Days post-inoculation

EDTA – Ethylenediaminetetraacetic acid

FFPE – Formalin-Fixed, Paraffin-Embedded

GHG – Greenhouse gas

IT – Infection thread

LB – Luria-Bertani

NF – Nod Factors

OD – Optical density

RBS – Ribosome binding site

RPM – Revolutions per minute

SDS – Sodium dodecyl sulfate

SM – Symbiosome membrane

TE – Tris-EDTA

TEG – Tris-EDTA Glucose

TY – Tryptone yeast

UAS – Upstream activator sequence

UMR – University of Manitoba *Rhizobium*

Chapter 1: Literature Review

1.1 Rhizobium and nitrogen fixation

Nitrogen is an essential element needed for forming the building blocks of life. It is a fundamental part of genetic materials, proteins, and other structural components of living organisms. Although nitrogen is plentiful in the atmosphere, it mostly exists in a form that is unusable by most living organisms. To incorporate nitrogen into biological systems, it must be converted into biologically accessible forms like ammonia (NH_3), nitrite (NO_2^-), and nitrate (NO_3^-). Several processes convert atmospheric nitrogen (N_2) into usable forms, including biological, atmospheric, and industrial nitrogen fixation. It has been estimated that roughly 380 teragrams ($1x10^9$ kg) of nitrogen annually (Tg N/year) are fixed through these processes (Galloway et al., 2008).

Diazotrophs are bacteria capable of converting nitrogen into biologically usable forms such as ammonia. They can live freely or form symbiotic relationships with other organisms. They fix atmospheric nitrogen (N_2) gas using nitrogenase, an enzyme that catalyzes the reduction of nitrogen gas into ammonia. This process is energy-intensive and can be described chemically as follows:

$$\mathbf{N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi}$$

Biological nitrogen fixation (BNF) by symbiotic diazotrophic bacteria is considered the largest source of fixed nitrogen in the nitrogen cycle, contributing about 230 Tg N/year (Galloway et al., 2008). It is estimated that roughly 60 Tg N/year is fixed in agricultural croplands (Herridge et al., 2008).

Among the diazotrophic bacteria, rhizobia are some of the most well-known and extensively studied organisms that form symbiotic associations (Gage, 2004; Lindström &

Mousavi, 2020). Traditionally, these bacteria were thought to be Alphaproteobacteria belonging to the order Hyphomicrobiales; however, more recently, it has been shown that Betaproteobacteria belonging to the family *Burkholderiaceae* can also form symbiotic interactions with leguminous plants. These are sometimes called β-rhizobia (Moulin et al., 2001). Collectively, these symbioses involve most of the 18,000 recognized legume species (Masson-Boivin et al., 2009). The result of this symbiotic relationship is the formation of a root nodule. Within the nodule, the plant provides an intracellular microoxic environment where the rhizobia reduce N_2 to ammonia (Appleby, 1984). This ammonia is then assimilated by the host plant (Pate et al., 1979).

Rhizobia tend to have large, multipartite genomes that often include mega plasmids and/or chromids (Jumas-Bilak et al., 1998; diCenzo & Finan, 2017; Geddes et al., 2020) . Most of the symbiotic functions of these bacteria are located in two separate gene clusters: the *nod* (nodulation) genes and the *nif* (nitrogen fixation) genes (Masson-Boivin et al., 2009). These are often found on extrachromosomal elements (Downie et al., 1983), or located on symbiotic islands on chromosomes. While this is a common pattern among most symbiotic nitrogen-fixing bacteria, there are some variations. For example, certain *Bradyrhizobium* species lacking *nod* genes have been reported to induce nodule formation on host plants (Giraud et al., 2007).

The initiation of a rhizobium-legume symbiosis occurs with a signal exchange between free-living rhizobia in the soil and the plant root. This exchange begins as legume plants release molecules such as flavonoids and betaines (Maxwell et al., 1989; Maxwell & Phillips, 1990; Gage, 2004). The specific molecule secreted by the plant determines which rhizobial species initiates the symbiosis (Allan Downie, 1994). These flavonoids are recognized by NodD transcriptional activator proteins, which trigger the activation of about 25 *nod* genes (Geddes &

Oresnik, 2016). The *nod* genes encode proteins involved in the synthesis and export of a lipochitooligosaccharide known as Nod factor (NF). Nod factors are chemical signals produced by rhizobia to communicate with the plant.

Nod factors consist of 4-5 residues of β-1,4-linked N-acetyl-D-glucosamine (GlcNAc) subunits that have an N-linked acyl tail attached at the nonreducing end (Gage, 2004). They are further modified by changes to the lipid tails and the addition of groups such as sulfate, acetyl, and fucose to the glucosamine residues (Spaink, 1994). These modifications vary among rhizobial species and provide plant specificity (Dénarié et al., 1996). Although it is common for each rhizobial species to interact with a specific legume, a single species can produce a variety of NFs and interact with multiple legume species (Perret et al., 2000).

The host plant senses NF via receptor-like kinases on its plasma membrane that possess extracellular chitooligosaccharide-binding LysM domains (Limpens et al., 2003; Madsen et al., 2003). The recognition of NF by receptor-like kinases triggers calcium (Ca^{2+}) spiking in the plant cell nucleus (Ehrhardt et al., 1996; Geddes & Oresnik, 2016). The integration of NF perception into a physiological response in the plant relies on this spiking. A Ca^{2+}/calmodulin-dependent protein kinase (CCaMK) present in plants decodes this Ca^{2+} spiking (Lévy et al., 2004), and CCaMK associates with the phosphorylated form of the protein CYCLOPS (or IPD3) (Limpens & Bisseling, 2014), which results in the activation of two transcription factors NSP1 and NSP2. The activated forms of these two transcription factors form a complex, that results in transcription of early nodulation genes (S. Hirsch et al., 2009; Middleton et al., 2007).

Once in proximity to the plant, rhizobia are entrapped by curled root hairs, which result from asymmetric growth at the root hair tip in response to NF (Heidstra et al., 1994; Esseling et

al., 2003). This interaction forms an apoplastic compartment between the bacterial cell wall and the plant (Catalano et al., 2004; Udvardi & Poole, 2013). The rhizobia trapped within the curled, colonized root hair (CCRH) start dividing, forming colonies called infection foci. The infection continues with a reversed polar growth of the root hair, leading to an invagination of the plasma membrane. As the invaginating plasma membrane moves further into the plant, it forms a new cell wall, forming what is called an Infection Thread (IT). Dividing rhizobia continuously colonize the IT as it advances into the inner cortical cells (Fournier et al., 2008). When rhizobia reach cells fated to be infected, those at the tip of the IT are endocytosed into the inner cortical cells. During this process, the released rhizobia are surrounded by a plant-derived membrane (Catalano et al., 2004). This membrane is known as the symbiosome membrane (SM). Exchange of all nutrients necessary for nitrogen fixation occurs across this membrane.

Nodule organogenesis results from complex signaling that leads to mature nodule formation. Mature nodules consist of a central zone, which consists of a mixture of infected and uninfected cells, surrounded by an outer cortex that consists of uninfected plant tissue that connects to the root vascular system (Udvardi & Poole, 2013). Root nodules can be either determinate (spherical) or indeterminate (elliptical). Whereas an indeterminate nodule exhibits persistent meristematic growth with continual bacterial infection occurring, determinate nodules do not have meristematic growth and have a defined lifespan (A. M. Hirsch, 1992; Sprent, 2007). Additionally it has been shown that rhizobia in indeterminate nodules tend to undergo a terminal differentiation whereas rhizobia within determinate nodules tend to remain viable (A. M. Hirsch, 1992; Mergaert et al., 2006; Fournier et al., 2008; Geddes & Oresnik, 2016).

1.2 Climate impact of Canadian Agriculture

The agriculture sector in Canada contributed 69 metric tons (Mt) of greenhouse gas (GHG) emissions in 2023 (*NIR_2023_Part1*, 2023). This represents 10% of the country's total emissions, marking a 48% increase since 1990. The primary gases emitted from agriculture are methane (CH_4) and nitrous oxide (N_2O). According to the inventory report, 76% of Canada's N_2O emissions in 2023 originated from agriculture, increasing from previously reported 45% in 1990. The main sources of N_2O in this sector are derived from the addition of inorganic nitrogen and biosolids as fertilizers; however, crop residue decomposition, animal manure, and crop management practices also contribute to overall emissions.

Focusing on emissions from agricultural soils, GHG emissions increased by 8 Mt to 18 Mt in 2023 from 1990 levels. This rise can be directly linked to the increased use of inorganic nitrogen fertilizers and the relative decrease in the proportion of nitrogen applied to perennial cropland. Specifically, emissions from inorganic nitrogen fertilizers alone reached 10 Mt in 2023, marking a 118% increase over the last three decades. The country also saw a 2.1 Mt increase in CO_2 emissions, a 262% rise since 1990, primarily due to the use of urea and urea ammonium nitrate. The same report states that 40% of the agricultural GHG emissions from Manitoba originate from direct and indirect N_2O emissions from soil.

The availability of nitrogen in agricultural fields restricts overall productivity. Recommendations from grower groups for fertilizer additions are based on empirical agronomic trials. However, the use of applied nitrogen fertilizer is inefficient, and much of the nitrogen is transformed by soil microbes into N_2O. Nodulated legume crops can acquire a significant proportion of their nitrogen needs through rhizobial nitrogen fixation. However, if a legume crop is nitrogen-limited due to poor or no nodulation, current practices suggest supplementing the

crop with nitrogen fertilizer. These cases could potentially be circumvented with the availability of a competitive and efficient nitrogen-fixing inoculant thereby, decreasing overall costs and GHG emissions.

1.3 *Rhizobium* as Biological Inoculant for Dry Beans

Dry beans (*Phaseolus vulgaris*), often referred to as common beans, are a legume crop grown and used for direct human consumption (Broughton et al., 2003). *P. vulgaris*, being of Mesoamerican origin (Bitocchi et al., 2012), was domesticated for human consumption from two distinct (and isolated) genetically differentiated gene pools, which are believed to have diverged from a common ancestor population more than 100,000 years ago (Gepts, 1988). Being low in fat, rich in protein, and having slowly metabolizable starch and fiber, it is a staple in many regions of the world (Reinprecht et al., 2020). Although *Rhizobium leguminosarum* bv. *phaseoli* is considered the standard inoculant for dry bean crops, *P. vulgaris* is considered a "promiscuous legume", because it is nodulated by a wide range of rhizobial species (Michiels et al., 1998). While most are from the genus *Rhizobium*, species of *Sinorhizobium*, *Bradyrhizobium* and *Paraburkholderia* have also been observed to nodulate this host (Dall'Agnol et al., 2016; Martínez-Romero, 2003; Motnenko et al., 2025).

Dry beans typically receive only 39% of their total N from symbiotic nitrogen fixation, and are considered poor nitrogen fixers compared to other legume species such as faba beans, soybeans, and lentils, which can acquire approximately 75% of their total N from root nodules (Dwivedi et al., 2015). Since the availability of nitrogen in soil is often the limiting factor for high yield in bean crops, nitrogen fertilizers are often used to fertilize beans plants. An example of such practice can be seen in the state of North Dakota in the United States, where the

recommendation for growing dry beans is to apply 70 lb N/acre without inoculation and 40 lb N/acre with inoculation (Franzen, 2023).

In controlled environments, inoculation of common beans with rhizobia has resulted in increased yield, biomass, and nitrogen levels (Samago et al., 2018). These results, however, do not translate to field conditions, as the *Rhizobium-Phaseolus* symbiosis has been observed to be less efficient in fields (Vargas et al., 2000). Since dry beans are widely consumed as a source of dietary protein, efforts are underway to identify efficient rhizobia for use as inoculum. The reality, however, is that there is a lack of commercially available strains, and that for those products that do exist, there is a lack of confidence in their efficacy. This has led growers to revert to growing dry beans with applied nitrogen.

1.4 Current Issues with Rhizobial Inoculants

When a rhizobial inoculant is used, it is expected that the crop plant will gain a net benefit through nodulation and subsequent nitrogen fixation. Rhizobia, however, are a common part of the rhizosphere community and can show great variation in their abundance as well as their genotypic composition in native soil environments (Yates et al., 2011). If an inoculant is applied and is outcompeted by native strains that fix nitrogen less efficiently, it reduces the benefits of SNF and can negatively impact crop yield and quality.

A study with soybeans reported that more than 10^3 rhizobia are needed on the seed, relative to the soil population, to see a response to inoculation (Weaver & Frederick, 1974). Other studies have shown that populations as low as 10^2 rhizobia per gram of soil can create a barrier to the inoculation response (Vargas et al., 2002). Additional research indicated that inoculation responses could occur even when the soils contained 10^3 (Hungria et al., 2003) and

10^4 (Hungria et al., 2000) rhizobia per gram. Overall, the results appear variable, but the theme of rhizobia competing for nodule occupancy remains consistent across all the studies. Therefore, for dry bean inoculation, the number of rhizobia cells and their ability to outcompete resident strains for nodule occupancy are considered key factors. Although competition for nodule occupancy is acknowledged as being important, and has been actively researched, understanding what makes rhizobia competitive from an applied perspective is poorly understood.

1.5 Rhizobia and Competition for Nodule Occupancy

Competition for nodule occupancy is considered a fundamental adaptive trait of rhizobia and plays an important role in selecting inoculants for agriculture. This trait has been studied extensively for developing inocula for legumes. Over the years, it has been observed that locally adapted indigenous rhizobia are much more effective at nodulating host legumes than inocula that are better at nitrogen fixation. As a result, the host plant is mostly colonized by inefficient native rhizobia. Triplett & Sadowsky (1992) refer to this as the "*Rhizobium* competition problem." As discussed in a review by Dowling (1986), competition for nodule occupancy depends on various factors, some related to the host plant, some from the infecting bacteria, and others from environmental or ecological influences. The focus of this discussion will be on the bacterial factors.

It's understood that competitiveness, like nitrogen fixation, is controlled by specific genes expressed at different times during nodule development (Ampe et al., 2003; Barnett et al., 2004). Therefore, the competitiveness of rhizobia is believed to be a genetic trait resulting from the combined actions of multiple genes (M. Mendoza-Suárez et al., 2021a). Competitiveness also appears to vary widely among individual strains (Aguilar et al., 2022). Onishchuck et. al.(2017) classified competition into two types: direct and indirect. Direct competition involves

mechanisms that directly prevent other cells from growing. An example of such competition is antibiosis. Trifolitoxin is a well-characterized small peptide antibiotic produced by *R. leguminosarum* bv. *trifolii* strain T24, initially discovered by Schwinghamer & Belkengren (1968). In the same study, they showed that this antibiotic was bacteriostatic against *S. fredii* strains and *R. leguminosarum* bvs. *phaseoli, trifolii,* and *viceae.* When the genes encoding this toxin, *tfx* (Triplett, 1988) were transferred from the non-nitrogen-fixing T24 strain into the genome of *R. leguminosarum* bv. *trifolii* TA1 (Triplett, 1990), the resulting strain exhibited increased nodulation competitiveness through direct competition in controlled environments.

Similarly, rhizobia have also been shown to carry loci that encode bacteriocins (Roslycky, 1967; Schwinghamer, 1975). These were originally categorized as small, medium, and large, based on the assumption that the size of the zone of inhibition caused by a strain with these traits was proportional to the size of the inhibiting molecule. Subsequently, it has been demonstrated that "small" bacteriocins are related to acyl-homoserine lactones (Gray et al., 1996; Schripsema et al., 1996; Blosser-Middleton & Gray, 2001; diCenzo et al., 2019), medium bacteriocins are called rhizobiocins and are connected to RTX-like hemolysins (Oresnik et al., 1999; Venter et al., 2001), and large bacteriocins are presumed to be defective bacteriophage particles (Lotz & Mayer, 1972; P. R. Hirsch, 1979). Genetically, it was shown that *R. leguminosarum* strains carrying rhizobiocins gained a competitive advantage in nodule occupancy.

Indirect competition involves using exploitative methods to compete, such as better uptake of limiting nutrients or utilizing the results of various biophysical characteristics. Being able to use different carbon sources to proliferate allows strains to better adapt to their environments, giving them a competitive edge.

Most of the work in this area has focused on genetics. Components involved in chemotaxis (Miller et al., 2007; Wheatley et al., 2020), motility (Caetano-Anollés et al., 1988), exopolysaccharide biosynthesis (Janczarek et al., 2009; Geddes et al., 2014), lipopolysaccharide biosynthesis (Niehaus et al., 1998; Campbell et al., 2002), and the breakdown of various carbon compounds (Oresnik et al., 1998; Ding et al., 2012; Vanderlinde et al., 2014) were reported to influence competition for nodule occupancy (M. Mendoza-Suárez et al., 2021b). Some determinants identified for one type of symbiosis were also found to affect competition in another. For example, glycerol was found necessary for competition in *R. leguminosarum* (Poysti & Oresnik, 2007; Ding et al., 2012) but not in *S. meliloti*. Similarly, erythritol influenced nodule competition in *R. leguminosarum* (Yost et al., 2006) but not in *S. meliloti* (Geddes et al., 2010). Most genetic determinants, such as those related to arabinose, galactose, amino acids, and homoserine, were only tested in one symbiosis, making broad conclusions difficult. Two determinants, the utilization of rhamnose and myo-inositol, have been shown to affect the ability of both *R. leguminosarum* and *S. meliloti* to compete for nodule occupancy (Oresnik et al., 1998; Galbraith et al., 1998; Fry et al., 2001; Rivers et al., 2022).

The ability to use rhamnose was first identified in *R. leguminosarum* bv *trifolii* as plasmid-encoded (Baldani et al., 1992). Further analysis of this locus showed that it consisted of two transcripts covering about 10 kb, which included both an ABC transporter and genes necessary for rhamnose metabolism (Richardson et al., 2004; Richardson & Oresnik, 2007; diCenzo et al., 2019).Transport mutants that could not utilize rhamnose were unable to compete with the wild-type strain for nodulation of clover plant roots. These mutants only competed equally with the wild type when present at ten times the wild-type level. Similar results were also observed when rhamnose breakdown was studied in *S. meliloti* (Rivers et al., 2022). However, in

S. meliloti, it was shown that mutants unable to utilize rhamnose also had a defect in root attachment (Rivers et al., 2022).

Work with *R. leguminosarum* bv. *viciae* mutants that have disruptions in genes responsible for *myo*-inositol catabolism showed that this had a greater impact on competitiveness for nodule occupancy compared to disruptions in genes responsible for *myo*-inositol transport. They concluded that the inability to compete with wild-type strains was not due to superior proliferation in the rhizosphere, but rather to displacement during infection (Fry et al., 2001). This suggests that the ability to catabolize *myo*-inositol during infection played a more significant role in competition than slower transport of myo-inositol.

A study conducted by Marchetti et al. (2010) demonstrates that the rhizobial success in colonizing the rhizosphere is driven by a multitude of factors, such as metabolic flexibility and environmental sensing. The evolutionary fate of good nitrogen fixers over poor fixers was also driven by the host plant sanctions in the root nodules (Marchetti et al., 2014), demonstrating the ability of good nitrogen fixers to survive in the rhizosphere. The improvement of nitrogen fixation using genetic methods as well as plant-adapted methods, therefore, seems to be an easier task to accomplish compared to adapting a strain to be more competitive overall. In that light, studying bacteria that are intrinsically competitive becomes an important factor to consider when aiming to produce a good inoculum for legumes.

Overall, for an inoculant strain to be considered competitive, it should possess genetic elements that allow it to effectively colonize the rhizosphere, prevent other bacteria from colonizing the host plant through direct or indirect competition, establish efficient symbiosis, and promote plant growth.

Over time, the study of assessing competitive strains has shifted from constructing these strains to screening for naturally occurring, intrinsically competitive strains native to specific regions or soil types. Many previous studies on competition experiments involved creating mutations and then competing the mutant with the wild type. The disadvantages of such methods include issues like the stability of introduced genes, the metabolic burden of these genes on the organism, and finding suitable recipient organisms for the cloned genes. Additionally, developing a strain that is competitive across most—if not all—agricultural environments is not realistic. Therefore, the focus has transitioned to identifying inoculant strains that are locally adapted and more region-specific. Studies such as those by Mwenda et al. (2023) are being increasingly favored because they concentrate on screening indigenous bacteria with competitive traits and explore methods for assessing competition in the rhizosphere. Techniques like stable reporter plasmid integration (M. A. Mendoza-Suárez et al., 2020) have been developed to visualize strains, facilitating the study of competition in non-sterile soil environments.

1.6 Objectives of the Study

There are no commercially available inoculants specific to dry bean (*P. vulgaris*) cultivation. Canadian bean growers, especially in the prairie provinces, rely on inorganic nitrogen fertilizers for growing dry beans. With the support of Manitoba Pulse and Soybean Growers, the lab received soil samples from various bean-growing regions of Manitoba. These samples were used for nodule trapping experiments. The results led to the collection of 168 rhizobia strains capable of nodulating *P. vulgaris*. These strains were purified, stored, and had their full genomes sequenced. To assess their nitrogen fixation ability, each of these 168 strains was inoculated onto dry bean plants grown under nitrogen-deficient conditions in a greenhouse trial. The study focused on the top 90th percentile of the strains tested in the greenhouse

experiment. The main goal of this research is to evaluate the competitive profile of *Rhizobium* spp. collected from agricultural soils in Manitoba, Canada.

The specific objectives are as follows:

1) Assess the competitiveness of wild-type *Rhizobium* strains
2) Characterize how the top competitive strains behave as a seed coating
3) Assess the effect of inoculant location, rhizosphere versus seed-coating, on root colonization and nodule occupancy using a reporter plasmid system.

Chapter 2: Materials and Methods

2.1 Bacterial strains, plasmids and media

Rhizobium strains used in the competition study are listed in Table 1. *Rhizobium* strains were grown at 28°C on Tryptone Yeast extract (TY), which consists of 5 g Tryptone, 3 g Yeast extract, and 0.5 g $CaCl_2$ per liter (Beringer, 1974). *Escherichia coli* strains were grown either at 28 °C (broth cultures) or at 37°C (agar plates) in Luria-Bertani broth containing 10 g Tryptone, 5 g Yeast extract, and 5 g NaCl (Sambrook et al., 1989). Agar media was solidified with 1.5% w/vol agar. For seed coating experiments, TY-broth was supplemented with sucrose to a final concentration of 10% w/v (Salema et al., 1982; Deaker, 2004). When necessary for auxotrophic strains, aminolevulinic acid (ALA) was added to a final concentration of 50 µg/ml, and Neomycin (Nm) was added to a final concentration of 40 µl/ml. All antibiotics and media supplements were filter-sterilized before addition. For long-term storage, isolated strains or strains containing plasmids were grown overnight in TY and mixed in a 2:1 ratio with a freezing solution containing 24% vol/vol dimethyl sulfoxide (DMSO) in TY, resulting in a final DMSO concentration of 8% vol/vol. These were then frozen and stored at -80 ºC.

2.2 Conjugation of barcoded plasmids into *Rhizbobium* isolates

Plasmids containing sequencing barcodes previously constructed (M. A. Mendoza-Suárez et al., 2020) were supplied in *E. coli* ST18. ST18 (Thoma & Schobert, 2009), which is a modified S17 λ*pir* strain (Simon et al., 1983) that does not carry a *hemA* gene, making it an aminolevulinic acid (ALA) auxotroph. The full genotype of ST18 is *recA, thi, pro, hsdR, RP4-2-Tc::Mu-Km Tn7/pir*, Δ*hemA*. This strain can mobilize plasmids for conjugation.

Table 1: List of *Rhizobium* strains used in competition experiments

Strain	Species	Point of Isolation (GPS Coordinates)
UMR 103	*R. hidalgonese*	49.497316, -98.045345
UMR 162	*Rhizobium.* sp.	49.6098445863, -100.1152153583
UMR 165	*R. anhuiense*	49.6098445863, -100.1152153583
UMR 201	*R. indicum*	49.5874524027, -99.8198049103
UMR 212	*R. leguminosarum*	49.5874524027, -99.8198049103
UMR 238	*Rhizobium.* sp.	49.6171178706, -99.8424809728
UMR 253	*R. redzepovicii*	49.6170872647, -99.8085271323
UMR 273	*Rhizobium.* sp.	MPSG-WP-4 (No GPS) *
UMR 275	*R. hidalgonese*	50.124783, -96.628944
UMR 278	*R. hidalgonese*	49.358667, -98.018561
UMR 279	*R. sophoriradicis*	2023WP-01 (No GPS) *
UMR 291	*Rhizobium.* sp.	49.993684, -98.348383
UMR 297	*R. croatiense*	49.129662, -97.67849
UMR 318	*R. anhuiense*	50.000743, -98.393761
UMR 330	*R. croatiense*	49.926764, -98.531525

UMR = University of Manitoba *Rhizobium*

(*): Proprietary field locations used by Manitoba Pulse and Soybean Growers

To carry out conjugations from ST18, both the donor *E.coli* and recipient *Rhizobium* strains were cultured on their appropriate solid media. Colonies of each strain were spread onto a 1 cm zone of TY-ALA agar by mixing with an inoculation loop. After overnight conjugation, the entire patch was streaked onto a TY-Nm plate. Nm^R transconjugants were purified three times and used immediately or stored at -80 °C for future use.

2.3 Plasmid extraction

Plasmid purification from *Rhizobium* strains was done using alkaline lysis. Strains were cultured till mid-log phase (OD_{600}= 0.8) in TY-broth as mentioned, and 3 ml culture was centrifuged at 5000 x *g* for 1 minute. The pellet was resuspended in 200 µl of Solution I (TEG). TEG solution contains 50 mM Tris (pH 8.0), 20 mM EDTA, and 1% glucose in 50 ml ddH_2O. 2 µl of RNase was added to remove RNA contamination. The mixture was homogenized by vortexing the tube for 30 seconds. 300 µl of Solution II (containing 1% SDS and 0.2 M NaOH) was added, and the tube was gently inverted 3 times to lyse the cells. Next, 300 µl of Solution III (3M Potassium Acetate, pH 4.8) was added to precipitate the chromosomal DNA and cell debris. The tube was gently inverted 3 times to mix all the components. This was then centrifuged at 10,000 x *g* in a microcentrifuge for 8 minutes, and the supernatant was pipetted into a new microfuge tube. Isopropanol (2-propanol) was added in a 1:1 ratio to the supernatant. The tube was then centrifuged at 10,000 x *g* for 15-20 minutes. After decanting the propanol, the plasmid pellet was washed with 400 µl of cold 70% EtOH followed by 95% ethanol. The ethanol was finally removed by gentle aspiration. Residual ethanol was removed by briefly centrifuging the

tube to concentrate it at the bottom. The residual ethanol was removed with a pipette, and the pellet was air-dried until there was no ethanol left. The pellet was finally resuspended in 20 µl ddH_2O.

2.4 PCR conditions

To carry out colony PCR, a small number of cells were taken from a fresh TY agar plate and resuspended in 20 µl of PCR reaction buffer. Colony PCR reactions were conducted using primers from Table 2. PCR reactions were carried out in a final volume of 20 µl. A small number of cells were collected from TY-agar plates and resuspended in a colony PCR reaction master mix. 100 µl of the master mix contained, 10 µl 10XC buffer [15.35 mM Tris-HCL pH 8.8, 10.05 mM KCL, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4 \cdot 7H_2O$, 0.1% v/v Triton X-100, 1.5 µM BSA] 2 µl dNTP (0.2 mM) , 2 µl forward primer, and 2 µl reverse primer (25 µM for each primer) , 1 µl Taq polymerase (house made Taq in 50mM Tris-HCL pH 7.9, 50mM KCL, 0.1 mM EDTA 0.5 mM PMSF, 50% v/v glycerol) and 83 µl ddH_2O. The PCR reaction conditions are as follows: primary lysis and denaturation at 95 °C for 10 minutes, followed by 25 cycles of 95 °C for 30 seconds, 57 °C for 30 seconds, and 68 °C for 30 seconds. The final elongation step was performed at 68 °C for 5 minutes.

PCR using plasmid DNA as template was done using the following recipe: For each 50 µl reaction, 10 µl of 10X buffer [120 mM Trish-HCL pH 8.0, 10mM KCL, 6mM $(NH_4)_2SO_4$, 0.1% v/v Triton X-100] , 1 µl dNTP (0.2 mM), 1 µl *taq* polymerase(house made taq in 50mM Tris-HCL pH 7.9, 50mM KCL, 0.1 mM EDTA 0.5 mM PMSF, 50% v/v glycerol), 1 µl DNA template(~80-120 ng), 1 µl forward primer and 1 µl reverse primer (25 µM for each primer).

Table 2: List of primers

Primer	Primer Sequence 5'-3'	Primer Name
	UMR 279 Specific	
1	CGGCAAGACGTTCGAAGTTC	279_pspA_F2_PS307 (Fwd)
2	CGTCATCACTTTTGCACCCC	279_pspA_R2_PS307 (Rev)
3	TGCCTTCTTCTGCGATGTGA	279_cetB_F1_P318 (Fwd)
4	GGTTCAACAACCAGGATTGGC	279_cetB_R1_P318(Rev)
	UMR 297 Specific	
5	ACGAGATCGAGAATGACGGC	297_neoG_F2_PS329 (Fwd)
6	CCTGCTGAACTGCCAGAAGA	297_neoG_R2_PS329 (Rev)
7	ATATCTCGACCACGTCACGA	297_PURA_F1_PS370(Fwd)
8	CAGATCATCTGCGATGGCCT	297_PURA_R1_PS370(Rev)
	ID- region amplification primers	
9*	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGCTACATCACGCATGGTATGGA	UFP_1 (Fwd)
10*	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGCTCCCCAGTCACGACGTTGTAAAACG	URP_1 (Rev)

(*): Primers adapted from (M. A. Mendoza-Suárez et al., 2020).

ddH_2O was used to make up a final volume of 50 µl. PCR conditions are as follows: 95 ºC for 5 minutes, then 25 cycles of 95 ºC for 30 seconds, 53 ºC for 30 seconds, 68 ºC for 30 seconds. Final elongation was done at 68 ºC for 5 minutes.

PCR amplification results were visualized on a 1% agarose gel using TAE buffer (40 mM Tris HCl, 20 mM acetate, 2 mM EDTA, pH 8.0) , using standard gel electrophoresis. The Invitrogen 1kb+ DNA Ladder was used for size references.

2.5 Genomic DNA extraction

Rhizobium strains were grown in TY-broth for 48 hours. Bacterial genomic DNA (gDNA) was extracted using the PureLink Genomic DNA Mini Kit (Invitrogen). Briefly, 1 ml of bacterial culture ($\leq 2x\ 10^9$ cells) was pelleted in a microfuge tube. The pellet was resuspended in 180 µl of PureLink® Genomic Digestion Buffer (proprietary recipe) and 20 µl Proteinase K. The mixture was vortexed to homogenize, then incubated in a 55 ˚C water bath for 90 minutes. After incubation, 20 µl RNase A (20 mg/mL enzyme in 50 mM Tris-HCl, pH 8.0, 10 mM EDTA) was added. The mixture tube was inverted 2-3 times and incubated for 2 minutes in room temperature. 200 µl of PureLink® Genomic Lysis/Binding Buffer was added to denature proteins and help the DNA bind to the columns in downstream steps. The recipe for the lysis/binding buffer is proprietary, but most genomic lysis/binding buffers contain 2-6 M guanidine chloride (Chirgwin et al., 1979), salt buffers, and surface active agents. 200 µl of 96-100% ethanol (EtOH) was added and mixed well. This mixture was then taken to the DNA-binding step.

Spin columns supplied with the kit have a reservoir capacity of 800 µl and can bind up to 500 µg of DNA, enabling purification of DNA in the size range of 25-50 kb. The ~640 µl

mixture from the previous step was added into a silica DNA binding column, and the column was placed on top of a collection tube. The tube was centrifuged at 10,000 x *g* using a microcentrifuge. The column was kept while the collection tube was discarded. The column containing genomic DNA bound to the silica matrix was placed into a new collection tube and taken to the wash step.

500 µl of Wash Buffer 1 (supplied by the kit) was added to the column, and the tube was centrifuged at 10,000 x *g* for 1 minute. The contents of the collection tube were discarded, and a second wash was performed with 500 µl of Wash Buffer 2 (supplied with the kit). The tube was centrifuged as before, and the collection tube was discarded. Most primary wash buffers contain 50-60% EtOH, ~125mM NaCl, ~1X TE (Rohland et al., 2010), while the second wash buffer usually contains a higher EtOH concentration (70-80%).

The column with the washed DNA was placed on a 1.5 ml microcentrifuge tube and 35-50 µl of ddH_2O was used to elute the DNA. Concentration of the DNA samples was done using a microplate reader (BioTek Epoch 2 Microplate reader) and A260/280 values were observed.

2.6 Sequencing library preparation

Nanopore library preparation was done using the Ligation Sequencing gDNA – Native Barcoding Kit 96 V14 (SQK-NBD114.96). Reagents were purchased from Oxford Nanopore, unless otherwise directed. DNA repair and end preparation was done using the NEBNext companion module from New England Biolabs.

The gDNA from all strains was quantified using a microplate reader (BioTek Epoch 2 Microplate reader). Samples were diluted and aliquoted into a 96-well PCR plate such that each

sample contained 400 ng of DNA. For each sample, 11 µl of gDNA, 0.875 µl of NEBNext FFPE DNA Repair Buffer, 0.875 µl Ultra II End-prep Reaction Buffer, 0.75 µl Ultra II End-prep Enzyme mix and 0.5 µl NEBNext FFPE DNA Repair mix were mixed to achieve a final volume of 15 µl. This DNA end-repair mix was incubated in a thermocycler at 20 ^{o}C for 5 minutes and then 65 ^{o}C for 5 minutes. A volume of 0.75 µl of each end-prepped sample was mixed with 3 µl nuclease free water, 1.25 µl of the Native Barcodes (NB 01- 96), and 5 µl of Blunt/TA Ligase Master Mix. Each sample was assigned its own barcode. The final 10 µl barcode mixture was incubated for 20 minutes at room temperature, and then 2 µl of the provided 0.25 M EDTA was added to each sample to stop the reaction. The barcoded samples were pooled into a single 1.5 ml DNA LoBind tube (Eppendorf DNA LoBind®). Keeping note of the total volume of the pooled samples, AMPure XP Beads were added at 0.4X of the total pooled volume. The pooled-magnetic bead sample was incubated at room temperature for 10 minutes. After incubation, the mixture was centrifuged briefly and placed on a magnetic rack to pellet the magnetic beads, until the supernatant appeared clear. The clear eluate was gently pipetted off without disturbing the magnetic bead pellet. The bead pellet was washed twice with 700 µl of 80% Ethanol (EtOH). The pellet containing the pooled and washed barcoded samples was then eluted in 35 µl of nuclease-free water at 37 ^{o}C for 10 minutes, with gentle agitation. The magnetic beads were then separated from the eluted DNA using a magnetic rack and the supernatant was removed.

The pooled-barcoded DNA sample was quantified using a Qubit and then processed further. 30 µl of the pooled sample was mixed with 5 µl of Native Adapter, 10 µl of NEBNext Quick Ligation Reaction buffer (5X) and 5 µl of Quick T4 DNA Ligase, resulting in a 50 µl mix. The mixture was incubated at room temperature for 20 minutes. Post incubation, 20 µl of the AMPure XP beads were added and washed as mentioned. For this washing step, instead of 80%

EtOH, 125 µl of Short Fragment Buffer (SB) was used. The DNA was eluted in 25 µl of nuclease-free water at 37 °C for 10 minutes. Depending on the expected size of the DNA fragments, the final elution volume was made up to be 32 µl as recommended by the manufacturer.

2.7 Library Sequencing

Libraries were sequenced using a PromethION 2 solo (Oxford Nanopore Technologies). This was carried out by first inserting the flow cells, determining the number of pores, using the MinKNOW software. Once the flow cell reached temperature and the pores were checked, the flow cell priming mix was added. To prepare the flow cell priming mix, 30 µl of Flow Cell Tether and 1.17 ml of Flow Cell Flush were mixed. After the P2 Solo was set up as per the user guide, 500 µl of the flow cell priming mix was added to the flow cell via the priming port. It is important to avoid air bubbles at this stage. After waiting for 5 minutes, 500 µl more of the priming mix was added. To prepare the library bead-prepared sample, 100 µl of Sequencing Buffer and 68 µl of Library Beads were mixed with 32 µl of adapter-ligated sample. After the priming step was done, 200 µl of the library bead-prepared sample was added into the flow cell using the inlet port using a P1000 pipette. Sequencing was carried out for 65 hours to achieve a 60x-100x coverage for all samples.

2.8 Bioinformatic analysis

2.8.1 **Assembly**

Basecalling was done with Dorado- High Accuracy (HAC) v1.1.1 model that is integrated in the Oxford Nanopore MinKNOW software. Quality filtering for the reads were done using

Fastp (Chen et al., 2018). Mean quality cutoff was Q20. Since HAC was used for base calling, length filtering was set to the default of 1000 bp. For each barcode, the reads generated were concatenated using bash command lines, and *de novo* assembly of the contiguous barcoded files was done using Flye (Kolmogorov et al., 2019),v2.9.6, setting the genome size at 7 Mb. The assembled genomes were analyzed using CheckM (Parks et al., 2015) v1.2.4. Assemblies that met the parameters of >95% completeness and <5% contamination were the ones being used downstream, as the final goal was to conduct a nucleotide-based match to an existing library. The genomes were also assigned to their taxonomic classification using the GTDB-tk v2.4.1, which uses the Genome Taxonomy Database (GTDB) (Chaumeil et al., 2020, 2022).

2.8.2 **FastANI analysis**

To conduct an Average Nucleotide Identity (ANI) analysis and create a dendrogram , FastANI v.1.34 (Jain et al., 2018) was run using ANIclustermap (Shimoyama, 2022) version 2.0.1 in a miniconda environment using bash command lines. Genomes of the *Rhizobium* spp*.,* collected from Manitoban agricultural soil*,* previously annotated in the Oresnik lab were used in the FastANI comparison to identify the newly run sequences. A sequence identity threshold of >98% was used for putative strain-level clustering.

2.8.3 **Pangenome Analysis**

To perform a pangenome analysis on selected strains, the FASTA files from the flye assembly were used to run Prokka (v1.14.6) (Seemann, 2014). Prokka annotates the sequences and automatically produces a set of files. The *.gff* files were then used to conduct a pangenome analysis with Roary (v 3.13) (Page et al., 2015), using the default 95% identity threshold for BLASTp. After generating a gene presence-absence matrix, an R script was employed to extract

all annotated and categorized genes unique to each strain into a single file, with each strain's genes organized in their respective columns.

Using the Genenious Prime software, the unique genes were located and curated from the library of annotated genomes. A custom BLAST database was created with sequences of the strains being used. Primers were designed to amplify regions within the unique genes, and then the custom BLAST database was used to check for primer off target matches. This was done in this manner, because the sequences being tested are unpublished, and so PrimerBLAST could not be utilized for this part. Primers tested or used for this study are listed on Table 2.

2.9 Colony enumeration

Colony counting was done using a modified version of the Miles & Misra method (Miles et al., 1938). For coated seeds, individual seeds were suspended in 1 ml of sterile water and vortexed thoroughly. 0.1 ml of that suspension was taken and diluted 10-fold. 10 µl drops from each dilution were plated onto TY-agar plates and incubated at 28 ºC for 24 hours. Three technical replicates were performed at each dilution point, and the average was used to calculate the average CFU/seed for each measurement. The colonies were visualized using a dissection microscope and a black background film to create contrast. The same procedure was used when enumerating colonies from liquid cultures: 0.1 ml of the culture was initially used for dilution to calculate CFU/ml.

2.10 Preparation of plant growth pots

Leonard Jar assemblies for general inoculation assays were done as previously described by Oresnik et al. (1994). The assembly was made from two magenta plant growth jars stacked on top of one another. The top jar had a cotton wick run through a hole drilled at its bottom. The top

jar was filled with a 1:1 w/w ratio of sand/vermiculite. The mixture was soaked with 250 ml of modified nitrogen-free Jensen's Solution (Jensen, 1942). The modified Jensen's solution contains: 1 g $CaHPO_4$, 0.2 g K_2HPO_4, 0.2 g $MgSO_4$, 0.2 g NaCl, and 0.1 g $FeCl_3$ in one liter of water. To this, 1 ml of trace elements were added. The trace elements solution contains: 0.5% (w/vol) H_3BO_3, 0.5% (w/vol) $MnSO_4$, 0.005% (w/vol) $ZnSO_4$, 0.005% (w/vol) Na_2MoO_4, and 0.005% (w/vol) $CoSO_4$.

Black pots with a volume of approximately 2.5 L were used for the multi-iteration competition experiment. The pots were sterilized using a 1 % bleach solution, followed by a 70% ethanol wash. The pots were then rinsed using water and dried inside the laminar flow cabinet. 1:1 w/w ratio of sand and vermiculite were autoclaved to sterilize the medium. The pots were filled up to 2/3 of their entire volume.

2.11 Seed preparation and inoculation

Navy Bean seeds were routinely used for all plant and seed assays. Seeds were surface sterilized using 1% bleach solution. The seeds were submerged in the bleach solution and gently agitated for 10 minutes. The bleach solution was then decanted. The seeds were then washed with 70% ethanol once. That was followed with washing with 10 times volume (compared to that of the bleach solution) of sterile water. After surface sterilizing the seeds were germinated on water agar plates (containing ddH_2O with 1.5% w/vol agar) in the dark at room temperature for 3-4 days. Germinated seeds exhibiting healthy primary root structures were aseptically planted into either sterile Leonard jar assemblies or 2.5 L pots. Planted assemblies/pots were subsequently covered with either a lid or plastic wrap and placed into a growth chamber. After the cotyledons appeared, the coverings were removed and the plants were inoculated with the *Rhizobium* strains. The chamber was maintained at 23 ^oC with 16 hours of light daily.

For single strain inosculations, each strain was grown in 5 ml TY-broth for approximately 2 days, to an OD_{600} of 1.0. 0.1 ml of the culture was added to 10 ml of sterile water and used to inoculate the seedlings. Overall, 10^8 cells were used to inoculate plants.

For multi-strain inoculations, the strains were cultured in broth and normalized to an OD_{600} of 0.5. A volume of 1 ml of each culture was mixed into a 15 ml conical tube and thoroughly vortexed to homogenize the mixture. From the tube containing the consortia, 0.1 ml of the consortia was mixed into 10 ml of sterile water and used to inoculate the plants. The pots were inoculated with approximately $5x10^7$ cells

2.12 Preparing Inocula from harvested nodules

To prepare inocula from harvested nodules, surface sterilized nodules were pooled into a single 15 ml conical tube and homogenized into a 10 ml slurry using sterile water. Tubes were centrifuged for 5 minutes at 150 × *g* in a swinging-bucket rotor. The supernatant was then carefully decanted into a fresh tube and was centrifuged at 10,000 rpm for 10 minutes to pellet the bacteroids. The bacteroids were then suspended in 5 ml of pre-vortexed TY-broth and recovered and enriched at room temperature overnight. The following day, newly germinated seedlings in new pots were inoculated with the overnight-recovered consortia.

2.13 Visualization of harvested roots, and bacteria purification from nodules

Post-harvest, plant roots were surface-sterilized by soaking in 1% bleach solution, followed by 70% ethanol. Then they were then washed twice in sterile water to remove the bleach solution. Surface sterilized nodules were then removed from the roots, crushed in 0.1 ml of sterile water; and streaked onto TY-agar plates. All isolates were single colony purified at least twice prior to being subsequently used.

Roots nodules containing rhizobia with the plasmid-IDs were visualized for fluorescence using Edvotek™ TruBlu™ 2 Blue/White Transilluminator. Root systems were placed inside the transilluminator and were exposed to 470 nm blue light. Nodules expressing the sfGFP protein glowed green.

2.14 Seed Coating

Bacterial strains were cultured overnight in a 5 ml broth. One ml of this initial culture was used to inoculate 250 ml of TY broth, which was then incubated for 2 days in a shaking incubator at 200 rpm and 28 °C. From this culture, 25 ml was centrifuged at 10,000 rpm for 10 minutes to pellet the cells. The cells were resuspended in 25 ml of TY containing 10% sucrose (vol/vol). Surface-sterilized seeds were soaked in the broth for 10 minutes. The medium was decanted, and the seeds were air-dried inside a laminar flow hood for 2-3 hours. Once dry, the seeds were stored inside a sterile petri dish at room temperature.

2.15 Rhizosphere Colonization

Rhizobium cultures were grown in the liquid media as described above until the late-log phase (OD_{600} 1.00). 25 milliliters of each culture were taken into a centrifuge tube and centrifuged at 10,000 rpm for 10 minutes. The media was decanted, and the pellet was resuspended in fresh TY broth. Appropriate dilutions were made to reach 10^7 CFU/ml, then mixed into autoclaved Leonard jar preparations presoaked in Jensen's solution. One milliliter of the dilution containing 10^7 cells was mixed with 50 ml of sterile water and poured into the sterile jar. The jars were kept at room temperature in the dark for 5 hours before planting seeds. Enumeration of cells on the seed coat and the rhizosphere was performed by drop dilution and counting.

Chapter 3: Results

3.1 Selecting *Rhizobium* isolates for competition

A successful inoculum strain should possess high nitrogen-fixation ability and be competitive for nodule occupancy. By screening approximately 50 soil samples provided by Manitoba Pulse and Soybean Growers, a collection of 168 rhizobial strains capable of nodulating common bean was previously isolated by members of the lab. To evaluate their nitrogen-fixation potential, each strain was inoculated onto bean plants in a greenhouse trial. The experiment included two replicates, each with a pot containing five bean plants inoculated with a *Rhizobium* culture and grown under nitrogen-deficient conditions, so that plant dry matter accumulation would reflect the total nitrogen fixed by the bacteria (Figure 1).

Since the goal of this work was to identify strains that were competitive for nodule occupancy, it was considered beneficial to focus on strains with already high nitrogen-fixation potential. To reduce the number of strains to be screened, it was decided that isolates in the 90th percentile would be the focus of this study. One isolate, UMR 278, which was not in the 90th percentile, was also included as a replacement for UMR 42, which was eventually considered as a contaminant *Bacillus* isolate and removed from the competition experiment. The 15 isolates and their collection locations are presented in Table 1.

3.1.1 Multi-iteration competition screen

The ability to find *Rhizobium* strains with superior nitrogen fixation is straightforward and involves either measuring nitrogenase activity or assessing plant dry weight when inoculated plants are grown under nitrogen-deficient conditions. Competition for nodule occupancy experiments has often relied on labor-intensive methods that can only process a few *Rhizobium* strains at a time (M. Mendoza-Suárez et al., 2021b). More recently, a few methods that utilize next-generation sequencing have been reported (Boivin 2020, Burghardt 2018).

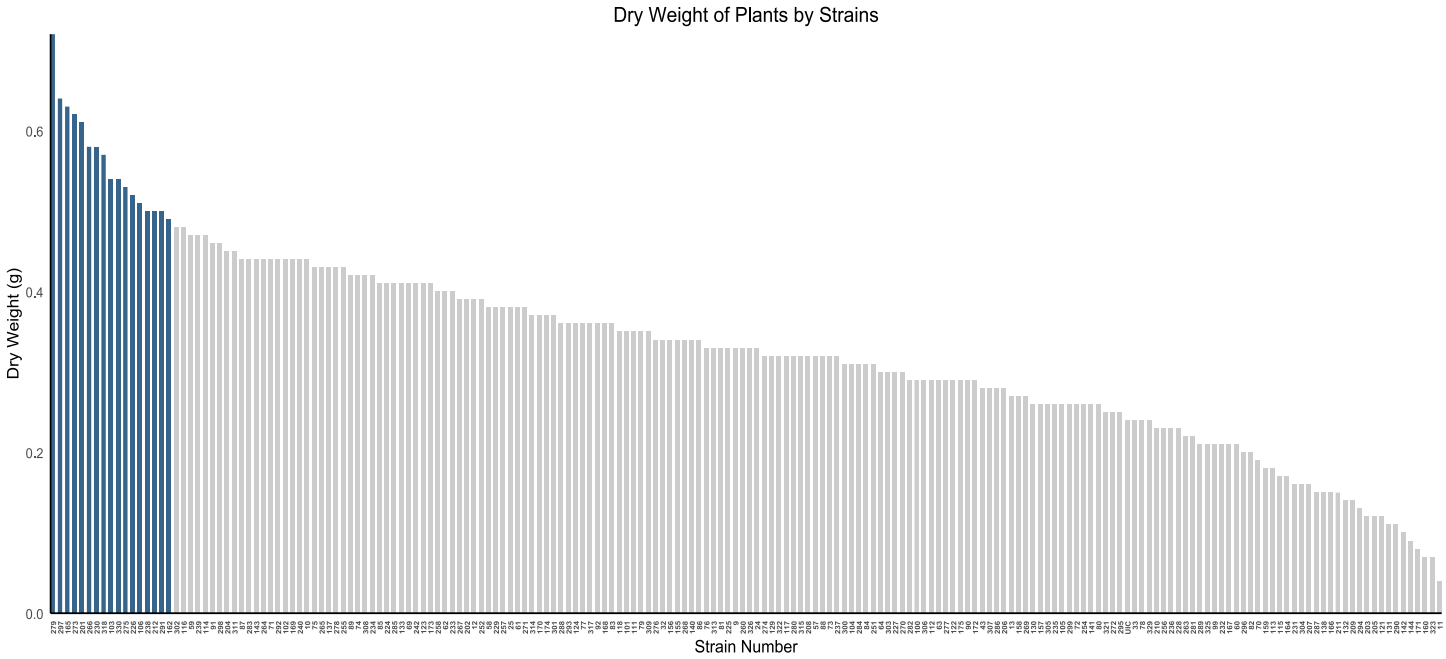

Figure 1: Nitrogen fixation profile of the 168 *Rhizobium* strains, using Shoot dry weight (SDW). The top 90th percentile is highlighted in blue. Navy bean seedlings were inoculated with a single *Rhizobium* strain and harvested 28 days after inoculation. Each strain was tested in two replicates. The lower of the two replicate values was taken as the representative value for each strain.

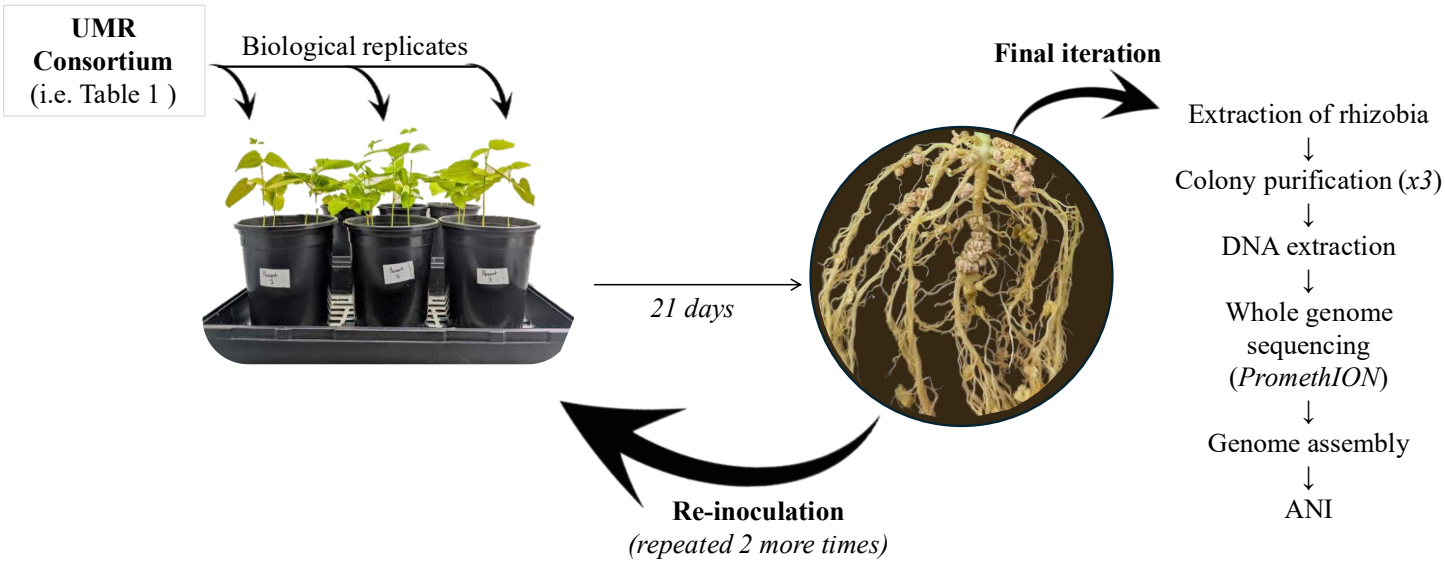

Figure 2: Overview of the multi-iteration competition experiment.
A consortium inoculant was created by combining 15 strains of *Rhizobium*, using OD_{600} values to normalize cells of each bacterial culture. Pots containing 4 plants were inoculated for each competition event. Roots were harvested after 21 days of inoculation and surface sterilized crown nodules were picked off. For the first two iterations, rhizobia were extracted from the nodules and used to reinoculated new pots. After the final iteration, rhizobia were extracted and colony purified from individual nodules and their genomic DNA were extracted. Whole genome sequencing, followed by Average Nucleotide Identity of the assembled genomes to the pre-established library of *Rhizobium* genomes was used to identify the sequenced strains.

Since the goal of this study was to screen many strains simultaneously, a strategy to achieve this was needed. The ability of rhizobia to quickly progress through the infection thread (IT) to establish early nodulation can be seen as a competitive trait; therefore, examining and identifying rhizobia that form nodules in the crown region of the roots can help find competitive inoculant strains (Burghardt & diCenzo, 2023; Cardoso et al., 2009). It was reasoned that, since the nodules at the crown were the oldest and first to develop, selecting these nodules could represent the most competitive strains within the consortium. To reduce the chance of less competitive strains randomly occupying these nodules, the isolated rhizobia from these nodules would be used as the next inoculum, thereby funneling the most competitive strains forward. Since each nodulation competition takes roughly 4 weeks to complete, the number of iterations was limited to 3, with 5 nodules from each of 4 plants selected for identification in the final iteration. Thus all of the isolated bacteria could be sequenced simultaneously on a single flow cell. An overview of the strategy is shown in Figure 2.

3.1.2 *Rhizobium* competition experiments

To conduct a competition experiment, plasmids containing the plasmid-IDs (referred to as bar-codes) (M. A. Mendoza-Suárez et al., 2020) were conjugated into each of the selected *Rhizobium* strains (Table 1). An inoculum consisting of the *Rhizobium* strains was normalized by optical density, and the prepared inoculum was poured into the center of the black competition pots, approximately equidistant from all four plants. Each black pot contained 4 plants and was a single competition event. The experiment consisted of 3 biological replicates. The experiment was also conducted using the same *Rhizobium* strains, but without the plasmids carrying the sequencing barcodes.

During the first two iterations of the competition experiment, the inoculated plants were grown for 21 days post-inoculation (DPI). After each harvest, the roots were sterilized. Following surface sterilization, five crown nodules from each plant, totaling 20 crown nodules from the entire competition pot, were collected. The 20 nodules from each competition were pooled, and this process was repeated for each competition. The bacteroids extracted from each pooled sample were used to inoculate a new set of pots. For the third and final iteration of the competition, the plants from both competition experiments (with and without barcodes) were harvested at 21 DPI, and the roots were surface-sterilized. Twenty crown nodules from each pot were collected, and the rhizobia from each nodule were isolated and purified.

The plasmids containing the unique sequencing IDs also carried a neomycin resistance cassette. Each purified isolate from the competition using the barcoded plasmids was screened on TY-Nm plates to confirm the presence of a plasmid. It was found that only 2 of the 60 purified strains carried Nm resistance, suggesting that although the plasmids were supposed to be stable (M. A. Mendoza-Suárez et al., 2020), they were lost during the competition experiment. To further verify the presence of the plasmid IDs, plasmids were extracted, and plasmid-ID-specific primers (Table 2) were used to amplify the ID region of the plasmid. Consistent with the screening results, no amplification bands were detected in any of the strains sensitive to Nm. Collectively, these data suggest that the plasmids were lost. As a result, the approach of using barcoded plasmids was abandoned for further competition experiments.

Rhizobium was isolated from a total of 60 crown nodules across three replicates that did not contain the barcoded plasmids. Genomic DNA from each isolate was extracted, and sequencing libraries were prepared and sequenced. After sequencing, the genomes were assembled. Out of the 60 isolates, 58 had successful sequencing runs.

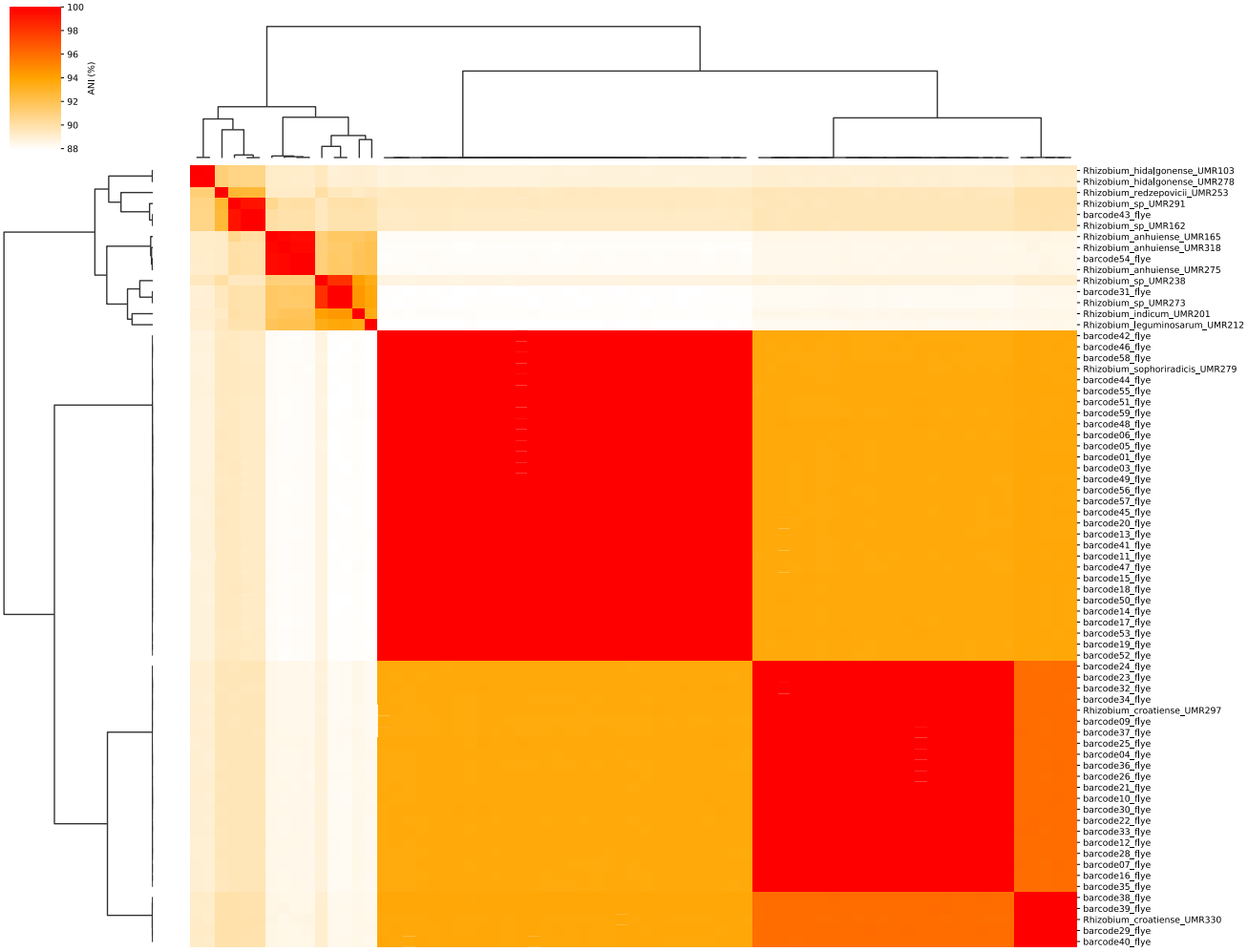

Figure 3: Average Nucleotide Identity (ANI) results from first competition experiment (Competitions 1-3)

The bacteria extracted from the nodules are labeled with their respective sequencing barcode numbers, whereas the strains labeled with their species name and UMR number respectively are the reference strains from the library of *Rhizobium* spp. collected from Manitoban soil

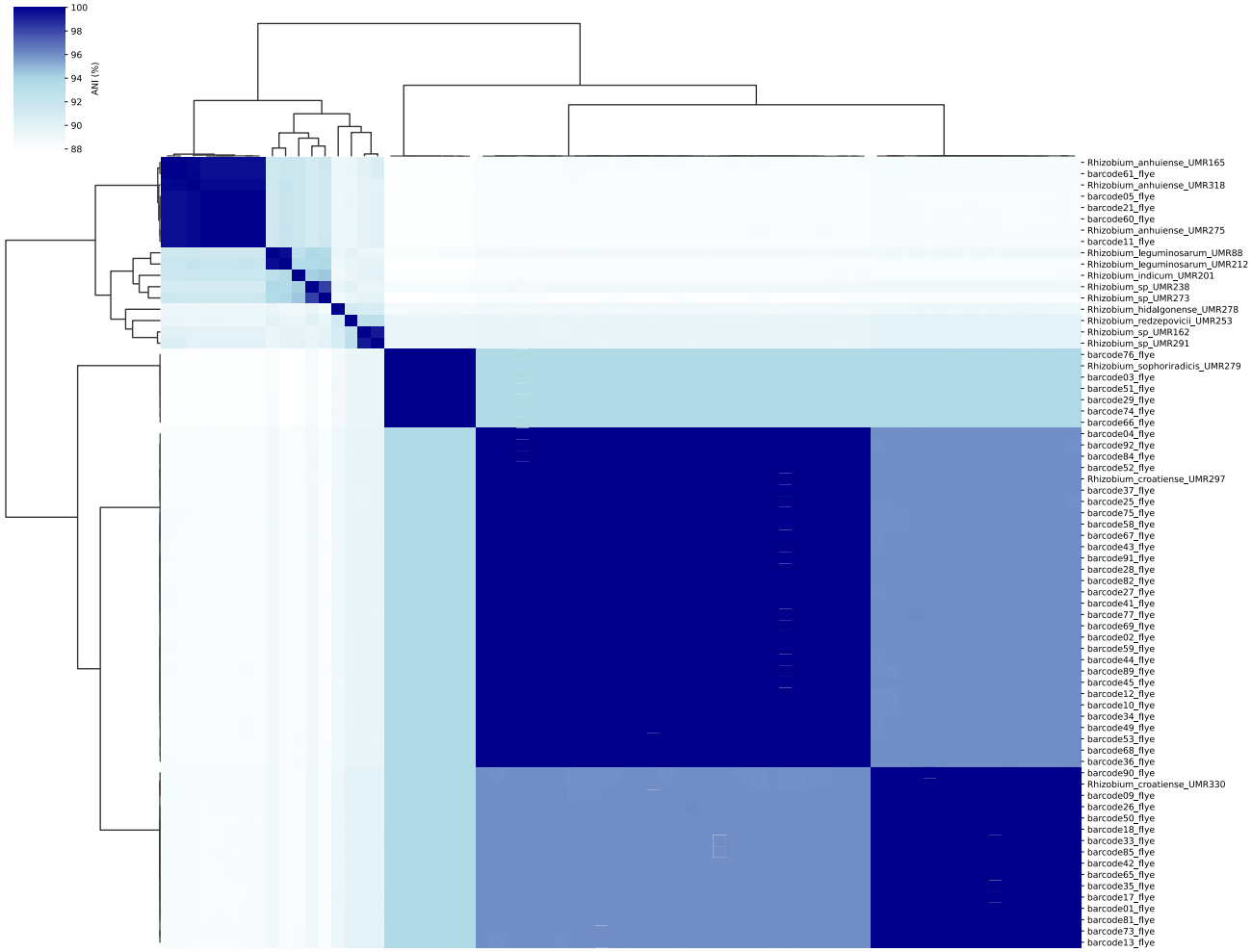

Figure 4: Average Nucleotide Identity results from Experiment 2 (Competitions 4-6)
The bacteria extracted from the nodules are labeled with their respective sequencing barcode numbers, whereas the strains labeled with their species name and UMR number respectively are the reference strains from the library of *Rhizobium* spp. collected from Manitoban soil

To identify the isolates, the average nucleotide identity of the entire genomes was compared with those of the input strains. The average nucleotide identity was calculated and visualized with using FastANI (Jain et al., 2018) which is integrated into ANIclustermap (Shimoyama, 2022). The results show that in the first three replicates, 7 out of 15 input strains were detected (Figure 3). These included UMR212, UMR238, UMR291, UMR318, UMR330, UMR279, and UMR297. The data also indicates that UMR279 was found in 53% of the crown nodules tested and in all three competition pots. UMR297 was detected in 36% of nodules and in two out of three replicates. Together, these strains accounted for 89% of all nodules tested. The remaining strains were detected in only one of the three replicates, representing 11% of the tested nodules. These included UMR 212, UMR 238, UMR 291, and UMR 330, which were present in two of the three pots.

To verify whether these results were reproducible, a second experiment with three independent replications was performed. The results indicate that, similar to the first experiment, a total of seven strains were present among the 60 harvested nodules. These strains included UMR165, UMR275, UMR291, UMR318, UMR330, UMR279, and UMR297 (Figure 4). UMR297 and UMR330 were the only strains found in all three replicates (Figure 5). The strains UMR297, UMR330, and UMR279 were detected in 47%, 25%, and 16% of all nodules, respectively. Together, these three strains made up 88% of all analyzed nodules. The remaining 12% was represented by UMR165, UMR275, UMR291, and UMR318.

When all six competitions were analyzed together, two clear winners emerged (Figure 6). In total, 120 crown nodules were examined and sequenced across six independent competitions.

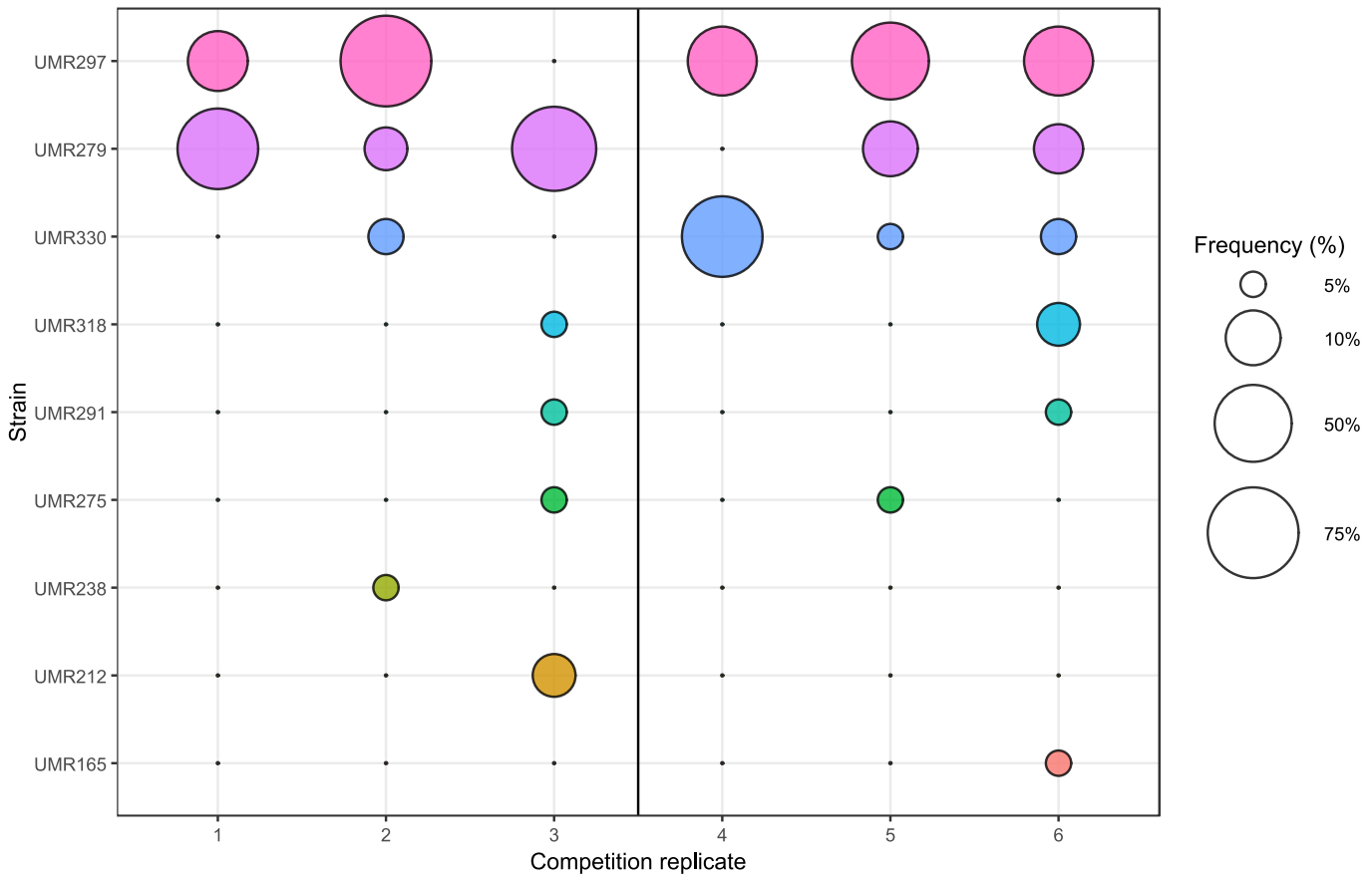

Figure 5: Strain frequency in each individual competition event.
The X-axis represents each individual competition pot replicate, where experiment 1 is competition replicate 1-3; and experiment 2 is 4-6. The two separate experiments are divided by the line break in the middle. The Y axis represents all 9 (out of 15) strains that were cumulatively found in both experiments. The strains are arranged in a descending order with respect to frequency. Each competition replicate contains 20 nodules from 4 plants meaning 5%, 10%, 50%, 75% would correlate to 1, 2, 10 and 15 nodules respectively,

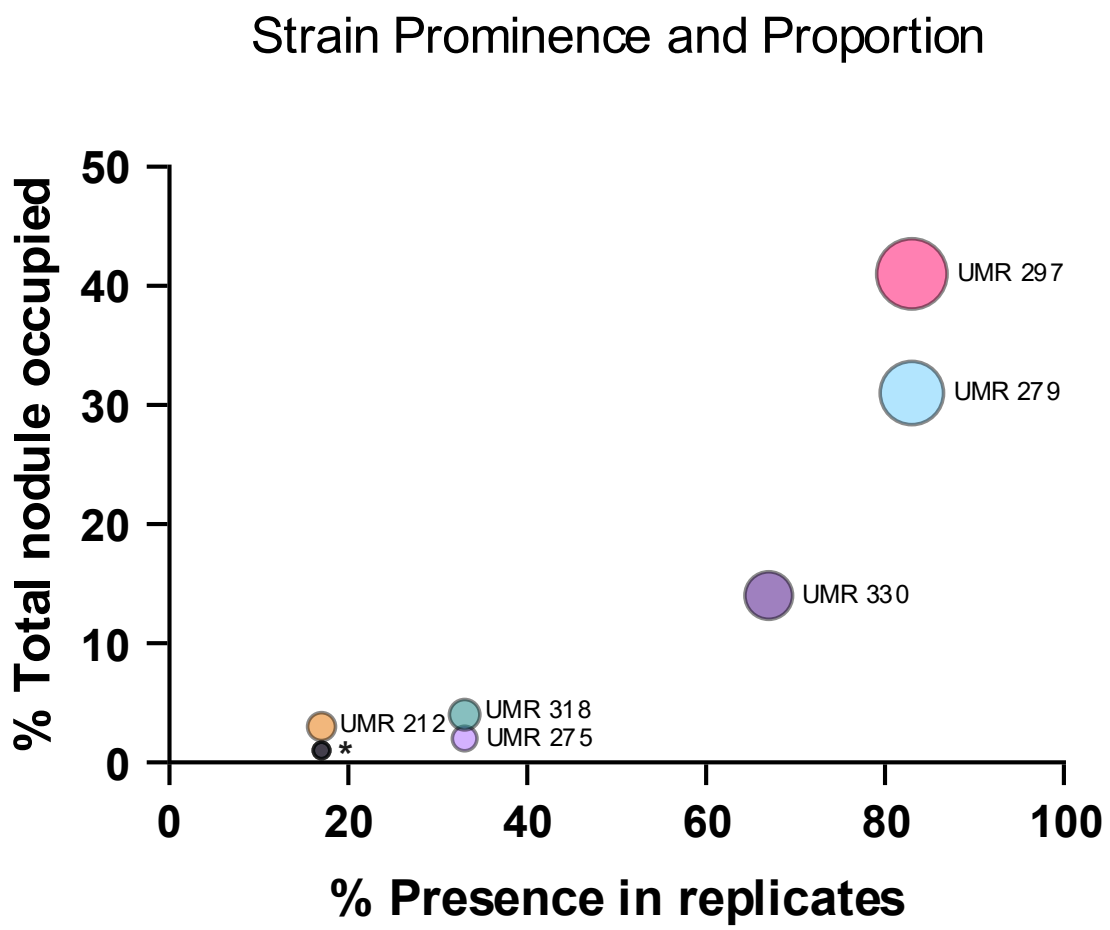

Figure 6: Cumulative strain prominence and proportion from all 6 competitions.
The Y-axis represents each strain's cumulative frequency, expressed as a percentage presence among all nodules examined. The X-axis represents the number of competitions replicates each strain was present in, expressed as a percentage presence. The size of each bubble denotes the frequency, meaning larger circles were present in the highest numbers in the most replicates. UMR 297 (pink bubble) and UMR 279 (blue bubble) were the top competitive strains, showing up in 41% and 31% of total nodules examined, and in 5 out of 6 competitions. (*)The small black circle shows the strains that were present in < 2% in total nodules occupied (UMR 165, UMR 238, UMR 291)

UMR 297 appeared to be the most competitive strain, present in 5 out of 6 replicates (83%) and accounting for 41% of all nodules tested. UMR 279 also appeared in 5 out of 6 replicates and made up 31% of all nodules tested. These two strains were considered the most competitive within the consortium. The remaining strains collectively represented 28% of the other nodules; among these, UMR 330 accounted for 15.9% out of the 28% , appearing in 4 out of 6 replicates (66%).

UMR 279 and UMR 297 were considered the most competitive because they had the highest numbers and appeared in most replicates. These successful strains were selected for further analysis.

3.2 Designing PCR primers specific for the top competitive strains

The primary goal of this work is to find strains suitable for field trials. Our method of identification, which uses whole-genome sequencing, works but is too expensive and time-consuming for regular use. To avoid the need to sequence rhizobia from nodules to identify the UMR strain of origin, we aimed to find strain-specific PCR primers.

To design strain-specific primers, whole-genome sequence files for strains UMR279, UMR297, UMR318, and UMR330 were used, and protein-coding regions were annotated using Prokka (v1.14.6)(Seemann, 2014). The output *.gff* files were used as input files to determine the pangenome using Roary (v3.13)(Page et al., 2015). The resulting gene presence-absence matrix was used to identify unique genes for each strain and was visualized as an UpSet plot generated by Complexupset v1.33 (Krassowski, 2020) (Figure 7). The results show that the 4 strains share 1,599 genes. Moreover, the analysis identified 5541 unique protein-coding regions in UMR318, 2259 in UMR279, 1483 in UMR297, and 1234 in UMR330 (Figure 7).

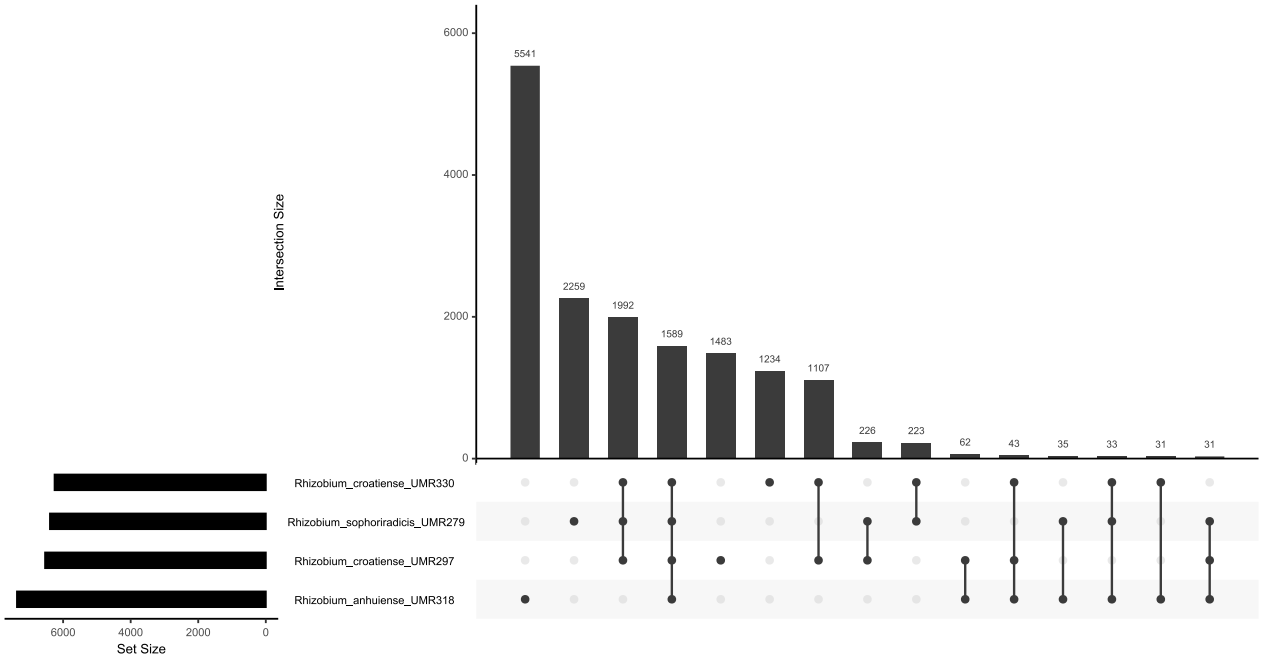

Figure 7: Pangenome analysis of some top competitive strains.
The UpSet plot depicts the distribution of unique and shared protein-coding genes across strains included in the pangenome analysis. The dot matrix defines gene intersections among strains, where connected dots represent shared gene sets and single dots indicate strain-specific genes. Vertical bars quantify the number of genes within each intersection, while horizontal bars represent the total gene count per strain. Further analysis was done using only UMR 279 and UMR 297.

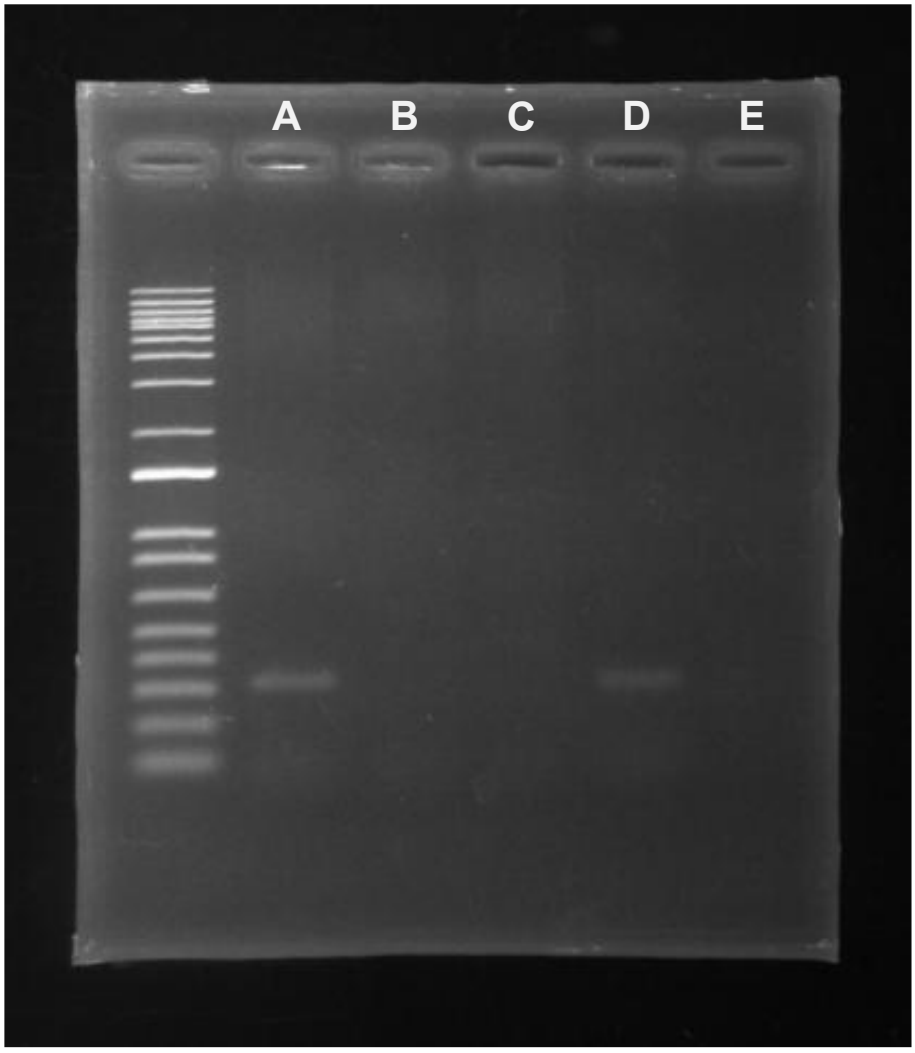

Figure 8: Gel image of bands obtained using finalized strain-specific primers.
Strain-specific genes were amplified using gene-specific primers to distinguish between UMR 279 and UMR 297. Lanes A–B correspond to reactions using UMR 279-specific primers, while lanes C–D correspond to UMR 297-specific primers. Lane E is a negative control lacking template DNA. Genomic DNA (gDNA) from UMR 279 was loaded in lanes A and C, and gDNA from UMR 297 in lanes B and D. Amplicons of the expected size (~300–400 bp) confirm the specificity of the primers and the presence of strain-specific genes. Primers 1-2 and 5-6 were used from Table 2.

Although 4 strains were used in the pangenome analysis, our immediate interest was in developing specific PCR primers to distinguish UMR279 and UMR297. From the identified genes, genes present on the chromosome were extracted into a list. A custom BLAST database was created in Genenious, and primers for the unique candidate genes were blasted against the whole-genome sequences of both strains to identify off-target binding (no primers with <2 mismatches off-target, including 2 mismatches within 3 base pairs of the 3' end). From this, two primer pairs from two unique chromosomal genes of each strain were selected based on similar amplicon size, Tm, GC content, lack of potential primer-dimer formation, and no off-target hits (Table 2).

To validate the primers, they were checked using both extracted genomic DNA as well as using colony PCR. One primer pair from each strain was finalized as the identification primers for the experiment based on band clarity on 1% agarose gel. The primer pairs were further optimized using a temperature gradient. The primer pairs chosen were 1+2 for UMR279, and 5+6 for UMR297. A representative gel showing unique products is shown in Figure 8.

3.2.1 Competing UMR279 and UMR297

The top 2 most competitive strains from the multi-iteration competition experiment were competed against each other in a single competition experiment. This was done to check how competitive the strains are against each other, when directly competed in a single iteration.

UMR 279 and UMR 297 were cultured in TY broth till late log phase, and optical density measurements (OD_{600}) were used to normalize both cultures. 1 ml of each culture was mixed into a single microfuge tube, homogenized and 0.1 ml of the mixed culture was used to inoculate seedlings. The inoculant contained approximately 1:1 ratio of cells from either culture roughly amounting 10^8 cells in each inoculation tube. Plants were harvested after 25 days post-

inoculation and nodules were collected from surface sterilized roots. The competition was conducted using 4 biological replicates. 24 nodules from each plant were harvested, and a total of 96 nodules were examined across the entire experiment. Rhizobia were cultured from each nodule and strain specific primers, custom made from a pangenome analysis of the two strains (Table2), were used in colony PCRs to identify which of the two strains were extracted from the nodules.

In 3 of the 4 biological replicates, UMR 279 & UMR 297 showed up in 0.29-0.33 (29%-33%) and 0.67-0.71 (67%-71%) proportions respectively. In one of the replicates, the proportion of UMR 279 was the greater value at 0.63 (63%). Figure 9 displays the observed outcome ratios for UMR 279 from all 4 replicates.

The input ratio of the strains was assumed to be 50:50 (input enumeration data lost), however without numbers for inoculation ratio, assumptions about the input ratio of the strains were tested against 10% standard deviation as well. Therefore, the following input ratios were hypothetically tested against the observed ratios UMR 279: UMR 297 in 50:50, 40:60, 60:40, 45:55 in the inoculum. This would account for deviations in cell numbers of strains in the inoculum and test for differences between expected versus observed ratios. A one sample t-test was conducted under the 4 scenarios with UMR 279 ratios, and p-values were noted to make assumptions about significance. Under all input conditions hypothetically tested against the observed data for UMR 279, p-values were above 0.05 ($p > 0.05$). In a 50:50 ratio, t-test resulted in a 0.28 p-value. For a (UMR 279: UMR297) 40:60, 60:40 and 45:55 ratio, p- values were 0.95, 0.08 and 0.54 respectively. So, an educated assumption can be made that both strains appear to be approximately equally competitive.

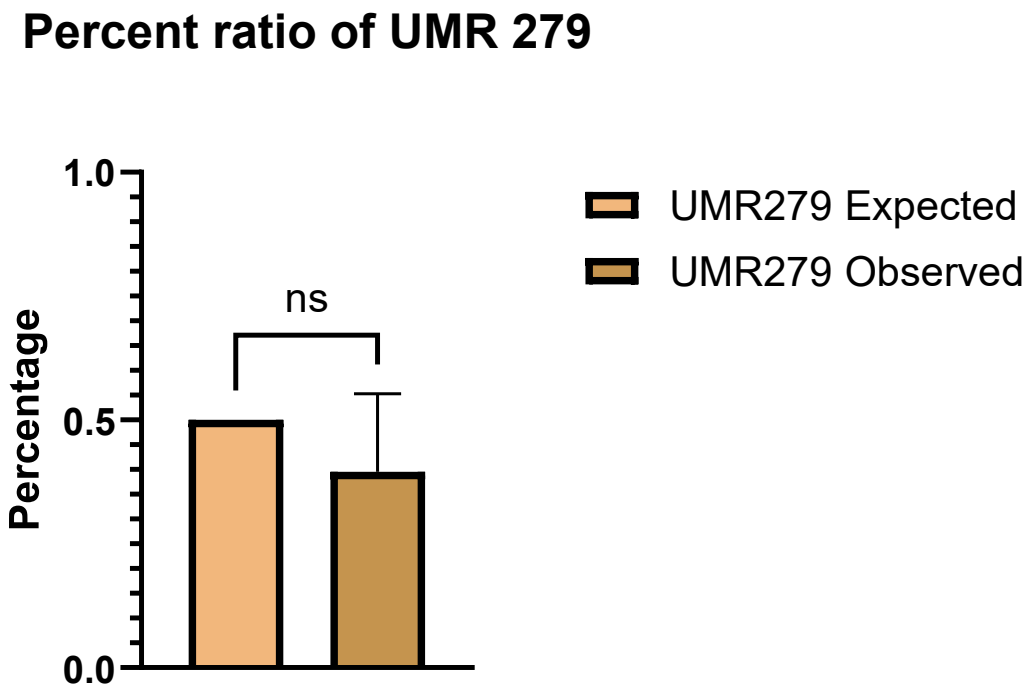

Figure 9: Expected versus observed ratios of UMR 279, in a 1:1 competition of UMR 279 and UMR 297.

Competition assays were performed by inoculating both strains at a 1:1 ratio. The bars on the x-axis are as noted in the figure. The y-axis (percentage), represents the percentage of UMR279 that was in either the inoculum or that was found in the nodules. A total of 96 nodules were collected from four plants (biological replicates). Rhizobia identified using strain-specific primers. Significance was determined using a t-test. ns, not significant.

3.3 Characterizing the survival of UMR279 and UMR297 on seeds

Inoculants for legumes come in various forms, including organic or inorganic carriers or applying the inoculant directly to the seed. Survival of an inoculant on carriers or seed coatings is a key factor in selecting inoculant strains (Roughley & Vincent, 1967). It has been previously noted that inadequate survival of the inoculant on the seed surface can negatively affect legume success (Trotman & Weaver, 1999). Since strains vary in their tolerance to desiccation, this is an important characteristic to consider when choosing a potential inoculant strain. Our goal is to test UMR279 and UMR297 under field conditions. To do this, we need a protocol for delivering the inoculum strains to the field. Given our available resources, we decided to first soak the bean seeds in *Rhizobium* resuspended in TY broth containing 10% sucrose (w/vol), then allow the mixture to dry onto the seeds. This approach was based on preliminary experiments conducted in my lab (Rai, 2019). My aim was to understand how UMR279 and UMR297 behave after application and drying on bean seeds.

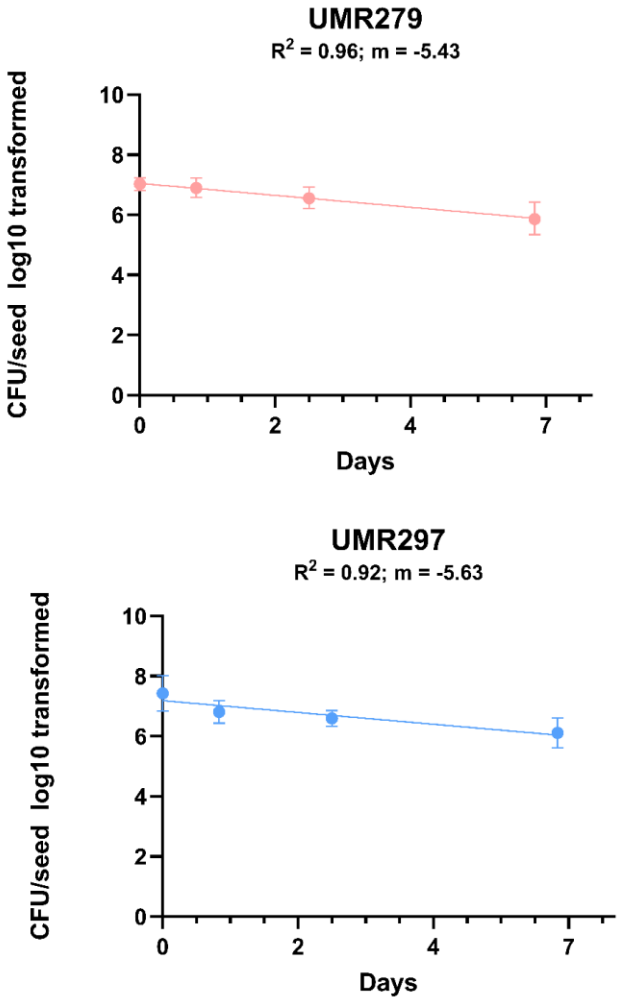

Figure 10: Survival of top competitive strains on seeds.
Seeds were coated by soaking the seeds with *Rhizobium* then air-drying the seeds. Top panel: UMR279. Bottom panel: UMR297. At the given time points, the number of viable cells on the seeds was determined. Each measurement represents the mean of 3 replicate measurements. The data was modeled using linear regression. R^2 values and the slope of the line (m) are noted above the graph.

3.3.1 *Rhizobium* viability declines over time when dried onto seeds.

Our initial question was to determine how long *Rhizobium* strains can survive on seeds. This was necessary to know since there would likely be a delay between treating the seeds and planting them. To find this out, strains were cultured to late log phase, pelleted, and then resuspended in TY broth containing 10% (w/vol) sucrose as an adhesive agent. Surface-sterilized navy bean seeds were submerged in the media and aseptically air-dried in a laminar flow hood. Cell viability was assessed by resuspending seeds in water, vortexing, diluting the suspension, and plating on TY agar.

In each case, the cultures that were used to soak the seeds had a titer of about 1×10^9 CFU/ml. The results show that immediately after seed coating, seeds typically contained approximately 10^7 colony-forming units per seed tested (Figure 10). Over 7 days, the numbers of viable UMR279 and UMR297 cells decreased in a predictable manner. Using linear regression, we see that the data can be fit to a straight line, with R^2 values of 0.96 and 0.92 for UMR279 and UMR297, respectively. The slopes of the lines (m) were also similar, with values of -5.43 and -5.63 for UMR 279 & UMR 297, respectively. From a practical perspective, we can conclude that seeds that have been coated with *Rhizobium* lose approximately one order of magnitude of viability over a period of a week.

3.3.2 Storage time appears to influence nodulation of coated seeds

Knowing that UMR279 and UMR297 can easily survive one week of desiccation on the seed coat is useful. What remains unknown is whether these desiccated cells are still capable of forming nodules. To determine this, cultures were dried onto seeds and planted over a 10-day time course. At each time point, some seeds were counted to determine the bacterial titer, while

Table 3: Viable cell counts of coated seeds at different days of storage

Day	Average log10 transformed CFU/seed	
	UMR279	UMR297
0	7.59±0.02	7.42±0.03
1	6.66±0.40	6.63±0.2
3	6.66±0.20	6.48±0.50
7	5.64±0.20	6.17±0.10
10	5.13±0.60	5.43±0.10

The values are an average ± standard deviation of 3 replicates measured for each day

Table 4: Number of nodules of coated seeds at different days of storage

Day	Average Number of Nodules	
	UMR279	**UMR297**
0	40 ± 4	44 ± 4
1	50 ± 2	30 ± 4
3	39 ± 2	36 ± 2
7	42 ± 5	34 ± 3
10	33 ± 4	31 ± 3

The values are an average ± standard deviation of 3 replicates measured for each day

Figure 11: Representative nodulated roots from seeds that were coated with UMR297. Seeds were coated with individual strains. Coated seeds were stored aseptically at room temperature over a period of 10 days. At each time point, seeds were planted in sterile sand–vermiculite supplemented with Jensen's solution. Nodulation was assessed by counting visible nodules (Table 3).

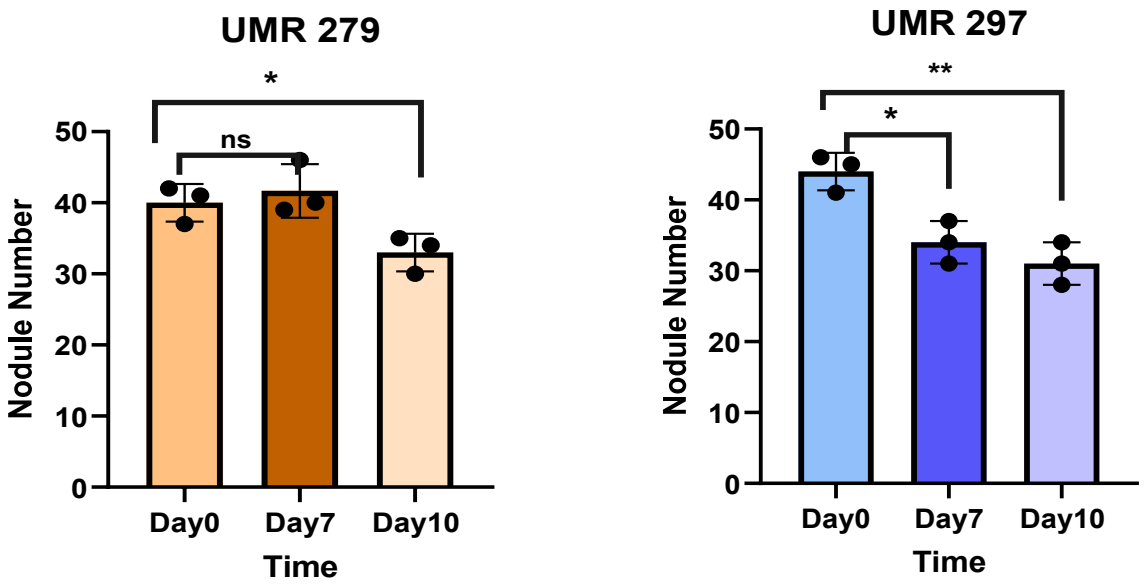

Figure 12: Comparison of nodule numbers of coated seeds from different time points. Seeds were coated with 10^7 cells, dried and stored at room temperature until planting. Nodules were counted post-harvest. Data for UMR 279 (*R. sophoriradicis*) is presented on the left. Data for UMR 297 (*R. croatiense*) is presented on the right. In all cases n=3. Significance was determined using a t-test. ns, not significant; *, $p < 0.05$; **, $p < 0.01$.

others were planted directly into sterile Leonard jar assemblies (Table 3). Plants were grown for 28 days after planting, and nodulated roots were visually examined.

A visual examination of the root systems developing from the coated seeds showed that seeds from all time points formed well-nodulated root systems with both crown nodules and nodules on the lower roots (Figure 11). The nodules were also counted (Table 4). It was observed that the number of nodules formed on day 0 was greater than the number formed from seeds stored for 10 days. To determine whether these differences were statistically significant, a two-tailed t-test was performed on nodule counts from the initial day of coating (Day 0) and from days 7 and 10 of the experiment.

Both strains showed a decrease in the number of nodules formed from the day of coating to the final day (Figure 12). UMR297 experienced a decline in nodule numbers, with a significant reduction from Day 0 to Day 10 ($p = 0.0051$). The other strain, UMR279, also showed a significant reduction on Day 10 ($p= 0.03$). However, the decline in nodule numbers differs between the two strains. Although both showed a similar pattern in cell viability during seed coating, UMR 297 exhibited a more linear and pronounced decrease in nodule count over time than UMR 279. The latter showed a more fluctuating profile, with a significant drop after Day 7. This indicates that viable cell count on the seed surface might not have a linear relationship with nodulation. It is worth noting that nodule number on a root system do not necessarily correlate with the amount of nitrogen fixed.

3.4 There is a positional advantage of rhizosphere cells in nodule occupancy

Previous studies have shown that *Rhizobium* from the rhizosphere can outcompete *Rhizobium* used as an inoculum (Moawad & Bohlool, 1984). Using a peat-based inoculum, strains of *Rhizobium* were marked with either *celB* or *gusA*. When stained with the appropriate

chromogenic substrates, nodules containing *Rhizobium* with these markers stain either blue (*gusA*) or purple (*celB*). It was found that when one strain is marked with either *gusA* or *celB*, one applied in the peat-based inoculum and the other in the rhizosphere, nodules were mostly formed by *Rhizobium* from the rhizosphere (Mwenda et al., 2023). Since we did not use a peat-based inoculum, we wanted to determine if the same outcome would occur under our seed-coating conditions. Additionally, rather than using the *gusA*/*celB* system that involves two different plasmids, we preferred to use the sfGFP system (Plasmid-IDs) so we could easily score the presence or absence of a particular strain based on fluorescence (M. A. Mendoza-Suárez et al., 2020).

As plasmid-IDs were not retained during the initial competition screening, a validation experiment was performed using previously conjugated and stored strains. Seeds were coated with plasmid-bearing strains and stored at room temperature for up to 8 days. At each time point, seeds were resuspended, serially diluted, and plated on non-selective TY media. One hundred colonies were screened on TY and TY-Nm plates (Table 5).

The results show that the strain used for this validation, UMR132 (pOPS0576), showed 100% retention across all time points, with all colonies growing on both media. A second strain, UMR207 (pOPS0554), exhibited 100% retention at day 0, with a 4% loss by day 8. Seeds were also sown at each time point and harvested 25 days post-inoculation; all recovered nodules exhibited fluorescence. These results indicate that plasmid-ID conjugated strains retain plasmids following seed coating and support their use as markers in subsequent rhizosphere colonization assays using the top competitive strains.

Table 5: Percentage of screened colonies of viable cells on seeds coated with Plasmid-ID tagged strains on TY-Nm selective media stored over time

Strain (Plasmid)	**Percentage of Patched Colonies on TY-Nm**		
	Day 0	**Day 4**	**Day 8**
UMR132(pOPS0576)	100	100	100
UMR207(pOPS0554)	100	98	96

Colony counts are expressed as the percentage of colonies recovered per 100 patched cells at Day 0, Day 4, and Day 8. Strain identity and species classification are indicated. Patching on non-selective TY medium yielded 100% recovery at all time points.

Table 6: Input number of viable cells on seed coating and the rhizosphere inoculation

Strain(plasmid)	Average log10_CFU/Seed	Average log10_CFU in Rhizosphere
UMR279	7.32[a]	7.34
UMR297	7.54	7.56
UMR279(pOPS0524)[b]	7.14	7.55
UMR297(pOPS0532)	7.13	7.41

[a] Data is presented as the average of three replicates

[b] Plasmid names indicate strains tagged with a plasmid-ID system with a sfGFP reporter gene (M. A. Mendoza-Suárez et al., 2020).

To perform this experiment, UMR279 and UMR297 were tested against themselves as seed coatings and as resident rhizobia within a simulated rhizosphere. To identify which strains originated from which position, the rhizosphere-resident counterparts were tagged with a plasmid carrying an sfGFP reporter. Since the sfGFP reporter system has not been used this way before, the experiment was designed to address variations caused by the plasmid by also conducting the reciprocal competition, in which the coated seeds contained the tagged strain, and the rhizosphere contained the untagged version of the same strain.

To simulate an established rhizosphere, sterile Leonard jar assemblies containing sand and vermiculite soaked with Jensen's solution were inoculated with either untagged or tagged (with plasmid-IDs) strains. Seeds were surface sterilized and also treated with tagged or untagged strains. The cell counts on the seeds and the rhizosphere were quantified to ensure equal numbers of rhizobia were present (Table 6). Seeds coated with untagged strains were sown into pots containing the tagged version of the same strain. Each combination was done in three biological replicates, and plants were harvested 28 days after sowing.

When the plants were uprooted, the root systems were initially visualized using blue light (Figure 13). Nodules were visualized and counted as either "glowing" or "dark" (Table 7). To determine the number of nodules formed from the strain in the rhizosphere, the proportion was calculated by dividing the total of either "glowing" or "dark" nodules, depending on whether the rhzosphere strain was tagged or untagged, by the total number of nodules on the root system (Table 7).

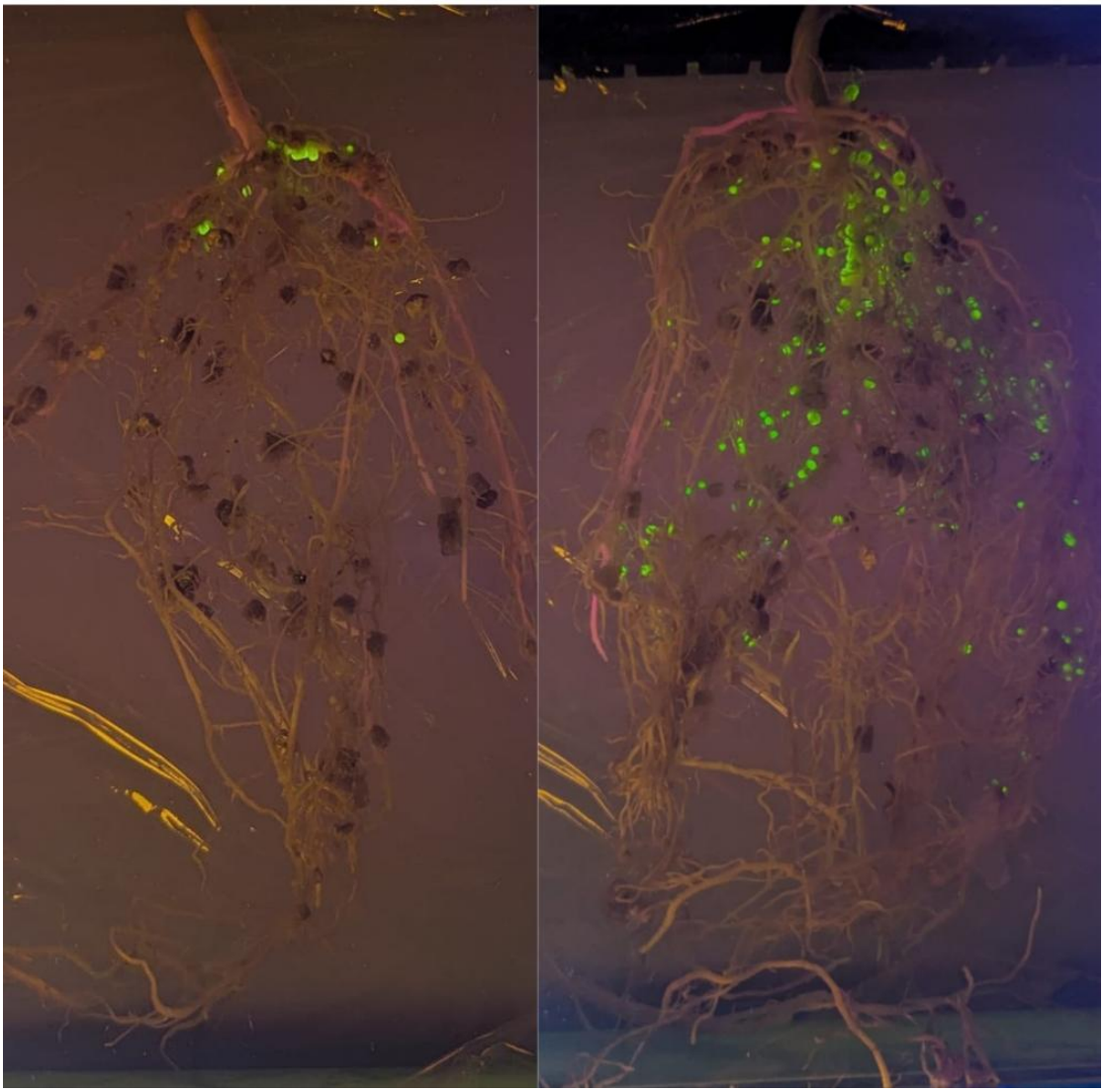

Figure 13: Representative images of a nodulated root system from a plant that had a tagged *Rhizobium* strain on either the seed (Left Panel), or in the rhizosphere (Right Panel). Note, square, non-fuorescent pellets visble on some roots are pieces of vermiculite that were not removed by gentle washing of the root.

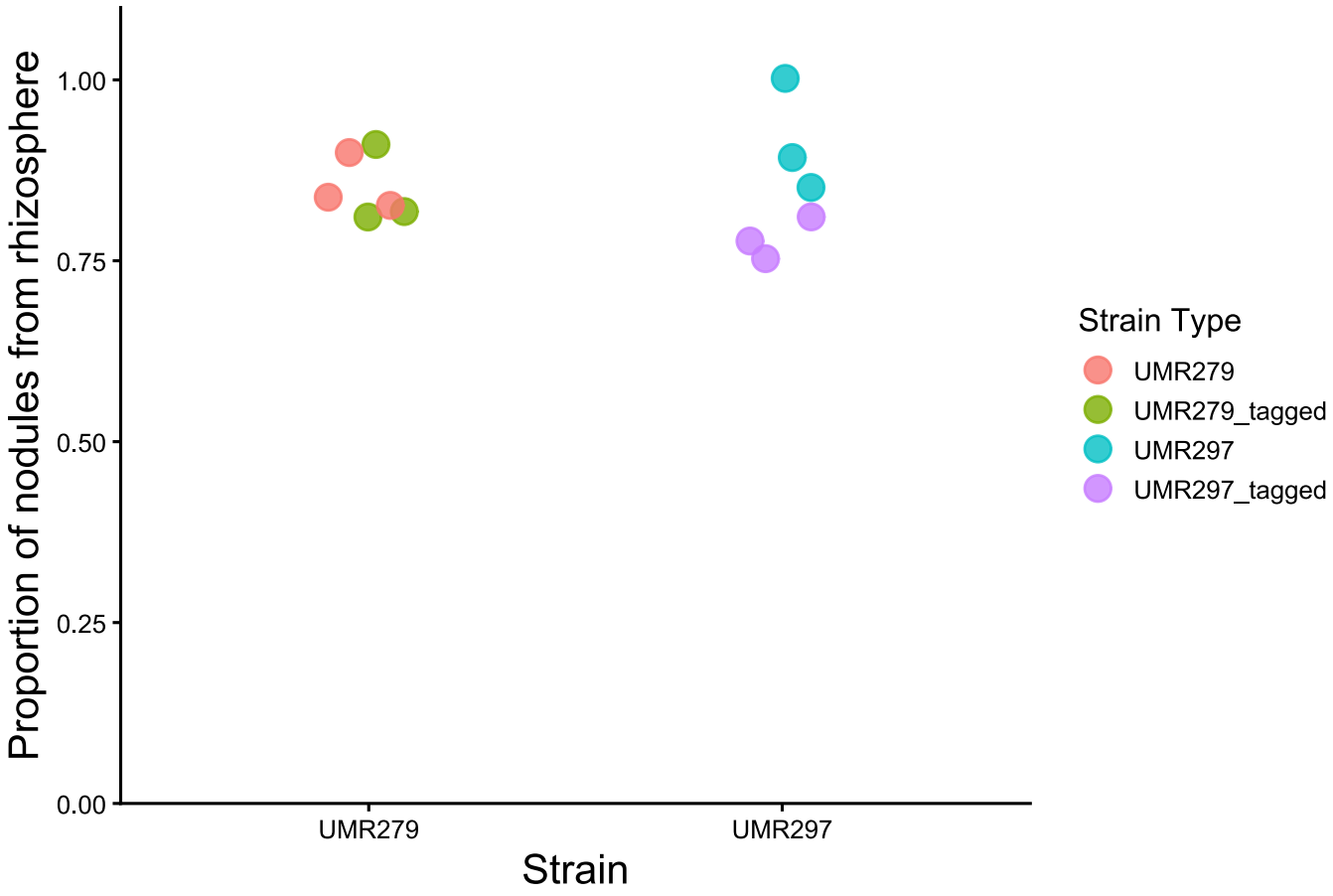

Figure 14: Proportion of nodules originating from strains in the rhizosphere
The proportion of nodules occupied by rhizosphere-inoculated strains was calculated relative to the total number of nodules per plant. These proportions demonstrate that both strains achieve higher nodule occupancy when introduced into the rhizosphere compared to seed coating, highlighting the influence of inoculation methods on competitive success.

Table 7: Nodule counts from the rhizosphere assay

Strain in Rhizosphere	Plant	Glowing Nodules	Dark Nodules	Total Nodules	Proportion from Rhizosphere[a]
UMR279_Tagged[b]	1	31	3	34	0.91
	2	28	6	34	0.82
	3	50	12	62	0.81
UMR279	1	3	28	31	0.90
	2	7	34	41	0.83
	3	6	32	38	0.84
UMR297_Tagged[c]	1	15	5	20	0.75
	2	35	8	43	0.81
	3	14	4	18	0.78
UMR297	1	0	20	20	1.00
	2	3	24	27	0.89
	3	3	17	20	0.85

[a] Proportion from the rhizosphere was calculated by dividing the number of nodules formed by the rhizosphere strain by the total number of nodules on the root system.

[b] UMR279 carrying the plasmid pOPS0524.

[c] UMR297 carrying the plasmid pOPS0532.

The results indicate that, under our experimental conditions, the proportion of nodules derived from rhizosphere cells ranged from 0.75 to 1.00, meaning that 75% to 100% of nodules from all examined roots originated from cells colonizing the rhizosphere (Table 7, Figure 14). UMR279 showed the least variation in proportion, ranging from 0.81 to 0.91 (81%-91%). This strain also produced the most nodules on the plant. UMR297 exhibited a larger variation among replicates, ranging from 0.75 to 1.00 (75%-100%). Interestingly, all nodules originating from the seed coating were observed only on the crown region, while nodules from the rhizosphere were found in both the crown and lateral regions of the root.

Chapter 4: Discussion

4.1 Adaptive competition screening

This study developed and optimized a screening method to select competitive *Rhizobium* strains from a consortium, aiming to identify strains suitable as inoculants for dry/common beans. No previous research has evaluated rhizobia's competitive ability through an adaptive, multi-iterative process, where the most competitive constituents in a multi-strain inoculant were enriched through consecutive inoculations

Rhizobia are facultative symbionts that inhabit competitive multi-strain environments, where they are subject to selection in both free-living and host-mediated contexts (Burghardt et al., 2018). While experimental evolution provides insights into bacterial fitness (Good et al., 2017), it does not directly reveal how natural genetic variations, such as communities of closely related strains like *Rhizobium* in this study, are selected. Developing synthetic communities of related bacteria helps evaluate the competitive fitness of genetic variants, supporting the identification of naturally competitive strains for inoculants.

Using multiple strains in a multi-iterative screening narrows the host plant's selection range. In legume-*Rhizobium* symbiosis, the plant host better indicates fitness than soil-based selection for nodulating rhizobia (Burghardt et al., 2018; Boivin et al., 2020). Emulating a multi-strain environment with an inoculant consortium and allowing the host to select the most competitive strain supports an adaptive screening protocol for future inoculants.

This thesis examines *Rhizobium* competitiveness, assuming biological differences among *Rhizobium* strains affect competitiveness. For example, strains UMR297 and UMR330, both *R. croatiense* from different locations (Table 1), show different competitive indices across six tests.

Only the top strains maintained significant proportions, while others remained below 10% in nodules. This raises questions about whether differences reflect true variability or chance.

A competition simulation was created assuming all strains had equal chances and that selection was random, simulating a multi-round experiment up to three rounds. This process was repeated 50 times, resulting in 1,000 nodules (20 per competition). The simulation showed that all strains had an average occupancy of 7%±1%, with a presence of 47%±8% in replicates (Appendix 2). When we compared this to the 6 real competitions, we see that most strains were below 6%, and nodule occupancy was close to their replicate frequency within the simulation. The exception was the top three strains, which had higher nodule occupancy and a higher prevalence across all replicates. Collectively, this suggests that the multi-iteration approach is identifying real biological differences.

4.2 Concordance between fixation and competitiveness

The screening method was developed using strains mostly in the 90th percentile for fixation efficiency from a greenhouse experiment, with UMR 278 as an outlier. Therefore, most strains were effective nitrogen fixers. Competition, a polygenic trait (M. Mendoza-Suárez et al., 2021a), differs from nitrogen fixation. Not all competitive strains are efficient fixers (Mwenda et al., 2023), as no correlation was found between fixation and competitiveness. However, some correlation has been observed between plant preference for “helpful” rhizobia strains and nitrogen fixation or biomass contribution (Burghardt et al., 2018)

The 9 (out of 15) strains found in nodules after all 6 competitions were plotted using their relative fixation rank (from a previous greenhouse experiment) and a competition rank based on strain frequency in nodules and replicates. The equation used is as follows:

$$\mathbf{CI} = \frac{\left(\mathbf{P_{nodules}} + \mathbf{P_{replicates}}\right)}{\mathbf{2}}$$

Where, CI = Competitive Index, $P_{nodules}$ = Percent presence in total nodules, $P_{replicates}$ = Percent presence in all replicates.

This was done to check for concordance between fixing nitrogen and competing for nodule occupancy (Figure 15). The data shows a link, but it involved a small subset and synthesized rankings, so the results are observational, not definitive.

4.3 Limitations of the screening

A study by (Burghardt et al., 2018) has shown that strain fitness does not correlate across different host genotypes and is not a predictor of selection fitness across various genotypes. Common beans come in various genotypes; however, as a first step toward developing a screening method using plant selection, all plant experiments in this study were conducted with Navy beans (AAC Shock variety – high-yield navy bean). The screening was performed in non-soil conditions, which allowed for the selection of rhizobia that are competitive without influences from their soil environment. However, in a real-world setting, the screening method should consider using soil to account for growth conditions in non-sterile fields.

Sequencing complete rhizobia genomes is expensive, so this study also used plasmid-ID (M. A. Mendoza-Suárez et al., 2020). These stable, conjugable plasmids contain unique DNA regions and use an sfGFP reporter under the nitrogenase promoter. Extracting plasmids from

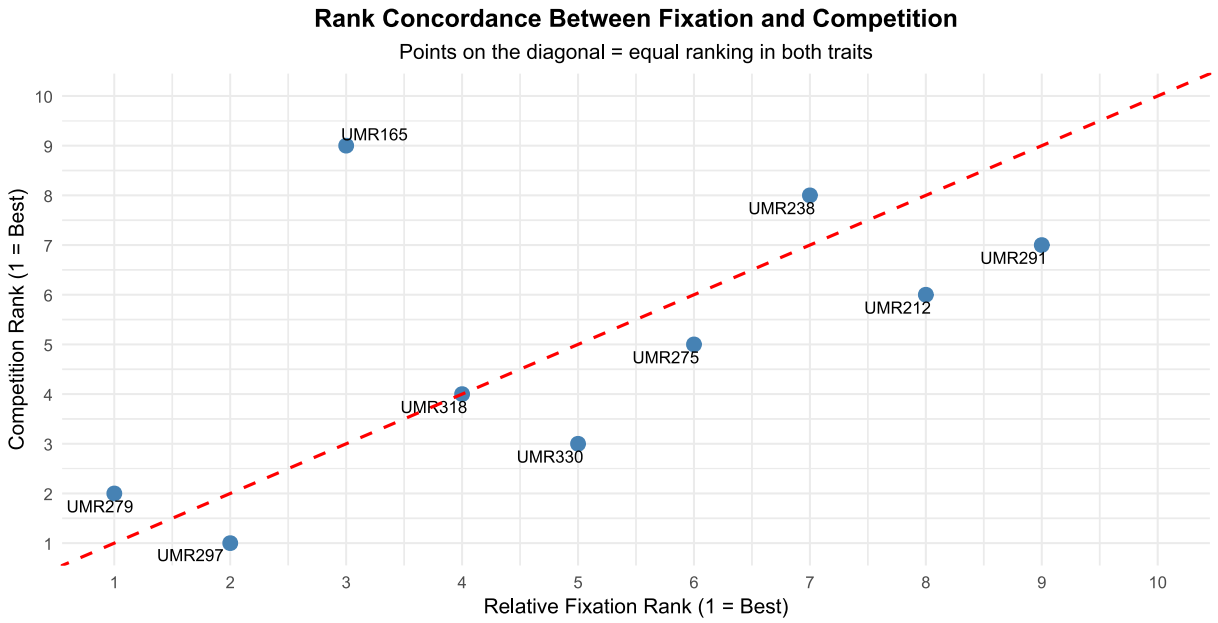

Figure 15: Rank concordance between nitrogen fixation and competitiveness.
Strains that were found at the end of the multi-iteration competition were ranked in two ways. The X-axis represents the relative fixation rank. The ranking of the SDW of plants inoculated with the strains in a greenhouse experiment were taken, and then the strains were ranked relative to themselves using the greenhouse data. The Y-axis shows competition ranking, done by averaging the sum of the percent presence in nodules and percent presence in replicates values from the competition. The data suggests concordance between the synthesized ranking. The strains closer to the origin of the graph represent strains that are both efficient fixers and competitive strains, representing strains that could have potential in inoculant development.

nodule rhizobia and sequencing the ID-amplicon significantly reduces costs. Single-colony isolation can sometimes remove extrachromosomal replicons (López-Guerrero et al., 2012). In multi-iteration tests, no plasmid retention was observed at the end (2 of 60 nodules). The loss may occur during extraction, re-inoculation, or colony purification but cannot be linked to a specific step. The method is adaptable to marker systems if the markers are distinguishable, such as different ID regions. Developing strain-specific primers was effective for fewer strains but might not work with large communities.

This study's screening doesn't consider nodules with multiple strains, as such nodules are lost during purification and sequencing. Between 1% and 20% of nodules may contain more than one strain (M. A. Mendoza-Suárez et al., 2020). The single colony method limits each nodule to one strain, usually the dominant. Using plasmid-ID systems, if they could be retained, might have addressed this by isolating DNA with an alkaline-PEG200 mixture for PCR amplification of ID regions (Chomczynski & Rymaszewski, 2006). Multiple IDs could have been linked to a single nodule during library prep. Nevertheless, this limitation isn’t critical for screening inoculant strains for mass production.

4.4 Limitations of counting nodule numbers

During the characterization of the top competitive strains used as seed coatings, it was observed that fewer nodules formed on roots from rhizobia-coated seeds that were kept at room temperature for several days. This procedure was undertaken to determine the optimal timing for coating seeds intended for field trials, especially when the coated seeds would be transported to farmlands or subjected to unfavorable weather conditions that could delay planting. Studies have shown a slight positive correlation between nodule number and nitrogen accumulation per plant (Kaziūnienė et al., 2025); however, a high nodule count does not always indicate increased

nitrogen fixation, as other factors also influence nitrogen fixation efficiency (M. Mendoza-Suárez et al., 2021a; Voisin et al., 2015). This study identified a significant difference in nodule numbers between plants grown from seeds coated with rhizobia on the initial day of coating versus those tested 10 days later. Nonetheless, shoot dry mass was not measured, so it remains unclear whether the reduced nodule numbers also led to lower dry mass in the plants. Assessing this in future experiments would be valuable to determine whether desiccation and storage time affect total nitrogen fixation, alongside changes in nodule count.

4.5 Considering effectiveness of seed coatings

In another characterization experiment, when the top strains from the competition experiment were used to determine whether the placement of the rhizobial inoculant provided a positional advantage for root nodulation, it was observed that the same strain was more likely to outcompete itself when in the rhizosphere rather than as a simple seed coating. It seems that even when strains in different positions in the pot were used in equal amounts, the strains resident in the rhizosphere held a positional advantage over the roots. Coating seeds with binders or carriers is a common practice in agricultural use of plant beneficial microbes (Rocha et al., 2019), and legume seeds are routinely inoculated through seed coating (Deaker, 2004). The findings from this study and that of Mwenda et al. (2023) offer insight into the impact of inoculation positioning and whether inoculation methods should be reconsidered for legume crops.

Chapter 5: Future Directions

The work in this thesis was done with the aim of developing and optimizing a screening method for potential dry bean inoculants. The screening method was successful in funneling a consortium into its most competitive constituents. This study is proof of concept that larger consortia of rhizobia can be competed against each other, to find an intrinsically competitive strain.

Competitive strain selection has been a challenge, especially for the screening of inoculants of *P. vulgaris*. The use of this screening method can allow for screening of hundreds of strains within one single experimental timeline. Strains that are found to be highly competitive for their host plant, but an ineffective nitrogen fixer can be further developed into an efficient fixer using adaptive evolution methods (Capela et al., 2017; Marchetti et al., 2010). The selection of universally competitive strains may not be a feasible idea. Soil types differ based on geographical location, and geographical location differences has been shown to have an impact on genetic evolution and adaptation (Greenlon et al., 2019). Therefore, this screening method can be further optimized for finding inoculants to be used in specific geographical locations using soil of said location as the matrix of competition.

Characterization of naturally competitive strains can help researchers further delve into the genetic mechanisms that constitute the competitiveness of rhizobia. The ability to screen large amounts of strains to find competitive rhizobia can allow for the establishment of a bank of competitive strains, which can further be competed in differing plant growth conditions (i.e. different soils, different levels of nutrients) to assess how the competitive profiles of the strains differ. Strains belonging to the same species showing vastly different

competitive profiles can be studied to see what genetic changes allow for such variance in competitiveness.

Studies regarding the positional advantage of rhizobia are important in shaping the correct method or carrier for dry bean inoculation. Being able to find competitive inoculant strains and decipher the delivery method of the strains could positively impact the inoculant practices for dry beans.

The strains deemed the most competitive in this study are scheduled to be tested in proper agricultural field conditions. This screening method will be applied in the workflow of screening for dry bean inoculants to be developed for Manitoban dry bean cultivation, under the BENEFIT project (Bio-inoculants for the promotion of nutrient use efficiency and crop resiliency in Canadian agriculture), by the University of Manitoba and Queen's University, among other universities in Canada.

Cited Literature

Aguilar, O. M., Collavino, M. M., & Mancini, U. (2022). Nodulation competitiveness and diversification of symbiosis genes in common beans from the American centers of domestication. *Scientific Reports*, *12*(1), 4591. https://doi.org/10.1038/s41598-022-08720-0

Allan Downie, J. (1994). Signalling strategies for nodulation of legumes by rhizobia. *Trends in Microbiology*, *2*(9), 318–324. https://doi.org/10.1016/0966-842X(94)90448-0

Ampe, F., Kiss, E., Sabourdy, F., & Batut, J. (2003). Transcriptome analysis of Sinorhizobium melilotiduring symbiosis. *Genome Biology*, *4*(2), R15. https://doi.org/10.1186/gb-2003-4-2-r15

Appleby, C. A. (1984). *Leghemoglobin and Rhizobium Respiration*.

Baldani, J. I., Weaver, R. W., Hynes, M. F., & Eardly, B. D. (1992). Utilization of Carbon Substrates, Electrophoretic Enzyme Patterns, and Symbiotic Performance of Plasmid-Cured Clover Rhizobia. *Applied and Environmental Microbiology*, *58*(7), 2308–2314. https://doi.org/10.1128/aem.58.7.2308-2314.1992

Barnett, M. J., Toman, C. J., Fisher, R. F., & Long, S. R. (2004). A dual-genome Symbiosis Chip for coordinate study of signal exchange and development in a prokaryote–host interaction. *Proceedings of the National Academy of Sciences*, *101*(47), 16636–16641. https://doi.org/10.1073/pnas.0407269101

Beringer, J. E. (1974). *Beringer1974*. Journal of General Microbiology.

Bitocchi, E., Nanni, L., Bellucci, E., Rossi, M., Giardini, A., Zeuli, P. S., Logozzo, G., Stougaard, J., McClean, P., Attene, G., & Papa, R. (2012). Mesoamerican origin of the common bean

(*Phaseolus vulgaris* L.) is revealed by sequence data. *Proceedings of the National Academy of Sciences*, *109*(14). https://doi.org/10.1073/pnas.1108973109

Blosser-Middleton, R. S., & Gray, K. M. (2001). Multiple *N* -Acyl Homoserine Lactone Signals of *Rhizobium leguminosarum* Are Synthesized in a Distinct Temporal Pattern. *Journal of Bacteriology*, *183*(23), 6771–6777. https://doi.org/10.1128/JB.183.23.6771-6777.2001

Boivin, S., Ait Lahmidi, N., Sherlock, D., Bonhomme, M., Dijon, D., Heulin-Gotty, K., Le-Queré, A., Pervent, M., Tauzin, M., Carlsson, G., Jensen, E., Journet, E., Lopez-Bellido, R., Seidenglanz, M., Marinkovic, J., Colella, S., Brunel, B., Young, P., & Lepetit, M. (2020). Host-specific competitiveness to form nodules in *Rhizobium leguminosarum* symbiovar *viciae*. *New Phytologist*, *226*(2), 555–568. https://doi.org/10.1111/nph.16392

Broughton, W. J., Hernández, G., Blair, M., Beebe, S., Gepts, P., & Vanderleyden, J. (2003). Beans (Phaseolus spp.) – model food legumes. *Plant and Soil*, *252*(1), 55–128. https://doi.org/10.1023/A:1024146710611

Burghardt, L. T., & diCenzo, G. C. (2023). The evolutionary ecology of rhizobia: Multiple facets of competition before, during, and after symbiosis with legumes. *Current Opinion in Microbiology*, *72*, 102281. https://doi.org/10.1016/j.mib.2023.102281

Burghardt, L. T., Epstein, B., Guhlin, J., Nelson, M. S., Taylor, M. R., Young, N. D., Sadowsky, M. J., & Tiffin, P. (2018). Select and resequence reveals relative fitness of bacteria in symbiotic and free-living environments. *Proceedings of the National Academy of Sciences*, *115*(10), 2425–2430. https://doi.org/10.1073/pnas.1714246115

Caetano-Anollés, G., Wall, L. G., De Micheli, A. T., Macchi, E. M., Bauer, W. D., & Favelukes, G. (1988). Role of Motility and Chemotaxis in Efficiency of Nodulation by *Rhizobium meliloti*. *Plant Physiology*, *86*(4), 1228–1235. https://doi.org/10.1104/pp.86.4.1228

Campbell, G. R. O., Reuhs, B. L., & Walker, G. C. (2002). Chronic intracellular infection of alfalfa nodules by *Sinorhizobium meliloti* requires correct lipopolysaccharide core. *Proceedings of the National Academy of Sciences*, *99*(6), 3938–3943. https://doi.org/10.1073/pnas.062425699

Capela, D., Marchetti, M., Clérissi, C., Perrier, A., Guetta, D., Gris, C., Valls, M., Jauneau, A., Cruveiller, S., Rocha, E. P. C., & Masson-Boivin, C. (2017). Recruitment of a Lineage-Specific Virulence Regulatory Pathway Promotes Intracellular Infection by a Plant Pathogen Experimentally Evolved into a Legume Symbiont. *Molecular Biology and Evolution*, *34*(10), 2503–2521. https://doi.org/10.1093/molbev/msx165

Cardoso, J. D., Gomes, D. F., Goes, K. C. G. P., Fonseca, N. D. S., Dorigo, O. F., Hungria, M., & Andrade, D. S. (2009). Relationship between total nodulation and nodulation at the root crown of peanut, soybean and common bean plants. *Soil Biology and Biochemistry*, *41*(8), 1760–1763. https://doi.org/10.1016/j.soilbio.2009.05.008

Catalano, C. M., Lane, W. S., & Sherrier, D. J. (2004). Biochemical characterization of symbiosome membrane proteins from ***Medicago truncatula*** root nodules. *ELECTROPHORESIS*, *25*(3), 519–531. https://doi.org/10.1002/elps.200305711

Chaumeil, P.-A., Mussig, A. J., Hugenholtz, P., & Parks, D. H. (2020). GTDB-Tk: A toolkit to classify genomes with the Genome Taxonomy Database. *Bioinformatics*, *36*(6), 1925–1927. https://doi.org/10.1093/bioinformatics/btz848

Chaumeil, P.-A., Mussig, A. J., Hugenholtz, P., & Parks, D. H. (2022). GTDB-Tk v2: Memory friendly classification with the genome taxonomy database. *Bioinformatics*, *38*(23), 5315–5316. https://doi.org/10.1093/bioinformatics/btac672

Chen, S., Zhou, Y., Chen, Y., & Gu, J. (2018). fastp: An ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics*, *34*(17), i884–i890. https://doi.org/10.1093/bioinformatics/bty560

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., & Rutter, W. J. (1979). *Isolation of Biologically Active Ribonucleic Acid from Sources Enriched in Ribonucleaset*.

Chomczynski, P., & Rymaszewski, M. (2006). Alkaline Polyethylene Glycol-Based Method for Direct PCR from Bacteria, Eukaryotic Tissue Samples, and Whole Blood. *BioTechniques*, *40*(4), 454–458. https://doi.org/10.2144/000112149

Dall'Agnol, R. F., Plotegher, F., Souza, R. C., Mendes, I. C., Dos Reis Junior, F. B., Béna, G., Moulin, L., & Hungria, M. (2016). *Paraburkholderia nodosa* is the main N_2 -fixing species trapped by promiscuous common bean (*Phaseolus vulgaris* L.) in the Brazilian 'Cerradão.' *FEMS Microbiology Ecology*, *92*(8), fiw108. https://doi.org/10.1093/femsec/fiw108

Deaker, R. (2004). Legume seed inoculation technology?a review. *Soil Biology and Biochemistry*, *36*(8), 1275–1288. https://doi.org/10.1016/j.soilbio.2004.04.009

Dénarié, J., Debellé, F., & Promé, J.-C. (1996). *Rhizobium Lipo-Chitooligosaccharide Nodulation Factors: Signaling Molecules Mediating Recognition and Morphogenesis*.

diCenzo, G. C., & Finan, T. M. (2017). The Divided Bacterial Genome: Structure, Function, and Evolution. *Microbiology and Molecular Biology Reviews*, *81*(3), e00019-17. https://doi.org/10.1128/MMBR.00019-17

diCenzo, G. C., Zamani, M., Checcucci, A., Fondi, M., Griffitts, J. S., Finan, T. M., & Mengoni, A. (2019). Multidisciplinary approaches for studying rhizobium–legume symbioses. *Canadian Journal of Microbiology*, *65*(1), 1–33. https://doi.org/10.1139/cjm-2018-0377

Ding, H., Yip, C. B., Geddes, B. A., Oresnik, I. J., & Hynes, M. F. (2012). Glycerol utilization by Rhizobium leguminosarum requires an ABC transporter and affects competition for nodulation. *Microbiology*, *158*(5), 1369–1378. https://doi.org/10.1099/mic.0.057281-0

Dowling, D. N. (1986). *Competition for Nodulation of Legumes*. *40*.

Downie, J. A., Ma, Q.-S., Knight, C. D., Hombrecher, G., & Johnston, A. W. B. (1983). Cloning of the symbiotic region of *Rhizobium leguminosarum*: The nodulation genes are between the nitrogenase genes and a *nifA* -like gene. *The EMBO Journal*, *2*(6), 947–952. https://doi.org/10.1002/j.1460-2075.1983.tb01526.x

Dwivedi, S. L., Sahrawat, K. L., Upadhyaya, H. D., Mengoni, A., Galardini, M., Bazzicalupo, M., Biondi, E. G., Hungria, M., Kaschuk, G., Blair, M. W., & Ortiz, R. (2015). Advances in Host Plant and Rhizobium Genomics to Enhance Symbiotic Nitrogen Fixation in Grain Legumes. In *Advances in Agronomy* (Vol. 129, pp. 1–116). Elsevier. https://doi.org/10.1016/bs.agron.2014.09.001

Ehrhardt, D. W., Wais, R., & Long, S. R. (1996). Calcium Spiking in Plant Root Hairs Responding to Rhizobium Nodulation Signals. *Cell*, *85*(5), 673–681. https://doi.org/10.1016/S0092-8674(00)81234-9

Fournier, J., Timmers, A. C. J., Sieberer, B. J., Jauneau, A., Chabaud, M., & Barker, D. G. (2008). Mechanism of Infection Thread Elongation in Root Hairs of *Medicago truncatula* and Dynamic Interplay with Associated Rhizobial Colonization. *Plant Physiology*, *148*(4), 1985–1995. https://doi.org/10.1104/pp.108.125674

Franzen, D. W. (2023). *Fertilizing Pinto, Navy and Other Dry Edible Bean*.

Fry, J., Wood, M., & Poole, P. S. (2001). Investigation of *myo* -Inositol Catabolism in *Rhizobium leguminosarum* bv. *Viciae* and Its Effect on Nodulation Competitiveness. *Molecular*

Plant-Microbe Interactions®, *14*(8), 1016–1025. https://doi.org/10.1094/MPMI.2001.14.8.1016

Gage, D. J. (2004). Infection and Invasion of Roots by Symbiotic, Nitrogen-Fixing Rhizobia during Nodulation of Temperate Legumes. *Microbiology and Molecular Biology Reviews*, *68*(2), 280–300. https://doi.org/10.1128/MMBR.68.2.280-300.2004

Galbraith, M. P., Feng, S. F., Borneman, J., Triplett, E. W., De Bruijn, F. J., & Rossbachl, S. (1998). A functional myo-inositol catabolism pathway is essential for rhizopine utilization by Sinorhizobium meliloti. *Microbiology*, *144*(10), 2915–2924. https://doi.org/10.1099/00221287-144-10-2915

Galloway, J. N., Townsend, A. R., Erisman, J. W., Bekunda, M., Cai, Z., Freney, J. R., Martinelli, L. A., Seitzinger, S. P., & Sutton, M. A. (2008). Transformation of the Nitrogen Cycle: Recent Trends, Questions, and Potential Solutions. *Science*, *320*(5878), 889–892. https://doi.org/10.1126/science.1136674

Geddes, B. A., diCenzo, G. C., Kearsley, J., Morton, R., & Finan, T. M. (2020). The genomes of rhizobia. In *Advances in Botanical Research* (Vol. 94, pp. 213–249). Elsevier. https://doi.org/10.1016/bs.abr.2019.09.014

Geddes, B. A., González, J. E., & Oresnik, I. J. (2014). Exopolysaccharide Production in Response to Medium Acidification Is Correlated With an Increase in Competition for Nodule Occupancy. *Molecular Plant-Microbe Interactions®*, *27*(12), 1307–1317. https://doi.org/10.1094/MPMI-06-14-0168-R

Geddes, B. A., Mendoza-Suárez, M. A., & Poole, P. S. (2019). A Bacterial Expression Vector Archive (BEVA) for Flexible Modular Assembly of Golden Gate-Compatible Vectors. *Frontiers in Microbiology*, *9*, 3345. https://doi.org/10.3389/fmicb.2018.03345

Geddes, B. A., & Oresnik, I. J. (2016). The Mechanism of Symbiotic Nitrogen Fixation. In C. J. Hurst (Ed.), *The Mechanistic Benefits of Microbial Symbionts* (Vol. 2, pp. 69–97). Springer International Publishing. https://doi.org/10.1007/978-3-319-28068-4_4

Geddes, B. A., Pickering, B. S., Poysti, N. J., Collins, H., Yudistira, H., & Oresnik, I. J. (2010). A locus necessary for the transport and catabolism of erythritol in Sinorhizobium meliloti. *Microbiology*, *156*(10), 2970–2981. https://doi.org/10.1099/mic.0.041905-0

Gepts, P. (1988). A Middle American and an Andean Common Bean Gene Pool. In P. Gepts (Ed.), *Genetic Resources of Phaseolus Beans* (Vol. 6, pp. 375–390). Springer Netherlands. https://doi.org/10.1007/978-94-009-2786-5_15

Giraud, E., Moulin, L., Vallenet, D., Barbe, V., Cytryn, E., Avarre, J.-C., Jaubert, M., Simon, D., Cartieaux, F., Prin, Y., Bena, G., Hannibal, L., Fardoux, J., Kojadinovic, M., Vuillet, L., Lajus, A., Cruveiller, S., Rouy, Z., Mangenot, S., … Sadowsky, M. (2007). Legumes Symbioses: Absence of *Nod* Genes in Photosynthetic Bradyrhizobia. *Science*, *316*(5829), 1307–1312. https://doi.org/10.1126/science.1139548

Good, B. H., McDonald, M. J., Barrick, J. E., Lenski, R. E., & Desai, M. M. (2017). The dynamics of molecular evolution over 60,000 generations. *Nature*, *551*(7678), 45–50. https://doi.org/10.1038/nature24287

Gray, K. M., Pearson, J. P., Downie, J. A., Boboye, B. E., & Greenberg, E. P. (1996). Cell-to-cell signaling in the symbiotic nitrogen-fixing bacterium Rhizobium leguminosarum: Autoinduction of a stationary phase and rhizosphere-expressed genes. *Journal of Bacteriology*, *178*(2), 372–376. https://doi.org/10.1128/jb.178.2.372-376.1996

Greenlon, A., Chang, P. L., Damtew, Z. M., Muleta, A., Carrasquilla-Garcia, N., Kim, D., Nguyen, H. P., Suryawanshi, V., Krieg, C. P., Yadav, S. K., Patel, J. S., Mukherjee, A.,

Udupa, S., Benjelloun, I., Thami-Alami, I., Yasin, M., Patil, B., Singh, S., Sarma, B. K., … Cook, D. R. (2019). Global-level population genomics reveals differential effects of geography and phylogeny on horizontal gene transfer in soil bacteria. *Proceedings of the National Academy of Sciences*, *116*(30), 15200–15209. https://doi.org/10.1073/pnas.1900056116

Herridge, D. F., Peoples, M. B., & Boddey, R. M. (2008). Global inputs of biological nitrogen fixation in agricultural systems. *Plant and Soil*, *311*(1–2), 1–18. https://doi.org/10.1007/s11104-008-9668-3

Hirsch, A. M. (1992). Developmental biology of legume nodulation. *New Phytologist*, *122*(2), 211–237. https://doi.org/10.1111/j.1469-8137.1992.tb04227.x

Hirsch, P. R. (1979). Plasmid-determined Bacteriocin Production by Rhizobium leguminosarum. *Journal of General Microbiology*, *113*(2), 219–228. https://doi.org/10.1099/00221287-113-2-219

Hirsch, S., Kim, J., Muñoz, A., Heckmann, A. B., Downie, J. A., & Oldroyd, G. E. D. (2009). GRAS Proteins Form a DNA Binding Complex to Induce Gene Expression during Nodulation Signaling in *Medicago truncatula*. *The Plant Cell*, *21*(2), 545–557. https://doi.org/10.1105/tpc.108.064501

Howieson, J. G., De Meyer, S. E., Vivas-Marfisi, A., Ratnayake, S., Ardley, J. K., & Yates, R. J. (2013). Novel Burkholderia bacteria isolated from Lebeckia ambigua – A perennial suffrutescent legume of the fynbos. *Soil Biology and Biochemistry*, *60*, 55–64. https://doi.org/10.1016/j.soilbio.2013.01.009

Hungria, M., Andrade, D. de S., & Probanza, A. (2000). *Isolation and characterization of new eÅcient and competitive bean (Phaseolus vulgaris L.) rhizobia from Brazil*.

Hungria, M., Campo, R. J., & Mendes, I. C. (2003). Benefits of inoculation of the common bean (Phaseolus vulgaris) crop with efficient and competitive Rhizobium tropici strains. *Biology and Fertility of Soils*, *39*(2), 88–93. https://doi.org/10.1007/s00374-003-0682-6

Jain, C., Rodriguez-R, L. M., Phillippy, A. M., Konstantinidis, K. T., & Aluru, S. (2018). High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. *Nature Communications*, *9*(1), 5114. https://doi.org/10.1038/s41467-018-07641-9

Janczarek, M., Jaroszuk-Ściseł, J., & Skorupska, A. (2009). Multiple copies of rosR and pssA genes enhance exopolysaccharide production, symbiotic competitiveness and clover nodulation in Rhizobium leguminosarum bv. Trifolii. *Antonie van Leeuwenhoek*, *96*(4), 471–486. https://doi.org/10.1007/s10482-009-9362-3

Jensen, H. L. (1942). *NITROGEN FIXATION IN LEGUMINOUS PLANTS. I. GENERALCHARACTERSOFROOT-NODULEBACTERIAISOLATEDFROMSPECIESOFMEDICAGO ANDTRIFOLIUMINAUSTRALIA. 67*, 98–108.

Jumas-Bilak, E., Michaux-Charachon, S., Bourg, G., Ramuz, M., & Allardet-Servent, A. (1998). Unconventional Genomic Organization in the Alpha Subgroup of the *Proteobacteria*. *Journal of Bacteriology*, *180*(10), 2749–2755. https://doi.org/10.1128/JB.180.10.2749-2755.1998

Kaziūnienė, J., Gegeckas, A., Lapinskienė, L., Razbadauskienė, K., Mažylytė, R., & Supronienė, S. (2025). Competitiveness and Nitrogen Fixation Efficiency Analysis of Rhizobium leguminosarum Strains in Different Field Pea (Pisum sativum L.) Genotypes. *Agriculture*, *15*(16), 1784. https://doi.org/10.3390/agriculture15161784

Kolmogorov, M., Yuan, J., Lin, Y., & Pevzner, P. A. (2019). Assembly of long, error-prone reads using repeat graphs. *Nature Biotechnology*, *37*(5), 540–546. https://doi.org/10.1038/s41587-019-0072-8

Krassowski, M. (2020). *ComplexUpset: Create Complex UpSet Plots Using "ggplot2" Components* (p. 1.3.3) [Dataset]. https://doi.org/10.32614/CRAN.package.ComplexUpset

Lévy, J., Bres, C., Geurts, R., Chalhoub, B., Kulikova, O., Duc, G., Journet, E.-P., Ané, J.-M., Lauber, E., Bisseling, T., Dénarié, J., Rosenberg, C., & Debellé, F. (2004). A Putative Ca^{2+} and Calmodulin-Dependent Protein Kinase Required for Bacterial and Fungal Symbioses. *Science*, *303*(5662), 1361–1364. https://doi.org/10.1126/science.1093038

Limpens, E., & Bisseling, T. (2014). CYCLOPS: A New Vision on Rhizobium-Induced Nodule Organogenesis. *Cell Host & Microbe*, *15*(2), 127–129. https://doi.org/10.1016/j.chom.2014.01.015

Limpens, E., Franken, C., Smit, P., Willemse, J., Bisseling, T., & Geurts, R. (2003). LysM Domain Receptor Kinases Regulating Rhizobial Nod Factor-Induced Infection. *Science*, *302*(5645), 630–633. https://doi.org/10.1126/science.1090074

Lindström, K., & Mousavi, S. A. (2020). Effectiveness of nitrogen fixation in rhizobia. *Microbial Biotechnology*, *13*(5), 1314–1335. https://doi.org/10.1111/1751-7915.13517

López-Guerrero, M. G., Ormeño-Orrillo, E., Acosta, J. L., Mendoza-Vargas, A., Rogel, M. A., Ramírez, M. A., Rosenblueth, M., Martínez-Romero, J., & Martínez-Romero, E. (2012). Rhizobial extrachromosomal replicon variability, stability and expression in natural niches. *Plasmid*, *68*(3), 149–158. https://doi.org/10.1016/j.plasmid.2012.07.002

Lotz, W., & Mayer, F. (1972). Isolation and Characterization of a Bacteriophage Tail-Like Bacteriocin from a Strain of *Rhizobium*. *Journal of Virology*, *9*(1), 160–173. https://doi.org/10.1128/jvi.9.1.160-173.1972

Madsen, E. B., Madsen, L. H., Radutoiu, S., Olbryt, M., Rakwalska, M., Szczyglowski, K., Sato, S., Kaneko, T., Tabata, S., Sandal, N., & Stougaard, J. (2003). A receptor kinase gene of the LysM type is involved in legumeperception of rhizobial signals. *Nature*, *425*(6958), 637–640. https://doi.org/10.1038/nature02045

Marchetti, M., Capela, D., Glew, M., Cruveiller, S., Chane-Woon-Ming, B., Gris, C., Timmers, T., Poinsot, V., Gilbert, L. B., Heeb, P., Médigue, C., Batut, J., & Masson-Boivin, C. (2010). Experimental Evolution of a Plant Pathogen into a Legume Symbiont. *PLoS Biology*, *8*(1), e1000280. https://doi.org/10.1371/journal.pbio.1000280

Marchetti, M., Jauneau, A., Capela, D., Remigi, P., Gris, C., Batut, J., & Masson-Boivin, C. (2014). Shaping Bacterial Symbiosis With Legumes by Experimental Evolution. *Molecular Plant-Microbe Interactions®*, *27*(9), 956–964. https://doi.org/10.1094/MPMI-03-14-0083-R

Martínez-Romero, E. (2003). Diversity of Rhizobium-Phaseolus vulgaris symbiosis: Overview and perspectives. *Plant and Soil*, *252*(1), 11–23. https://doi.org/10.1023/A:1024199013926

Masson-Boivin, C., Giraud, E., Perret, X., & Batut, J. (2009). Establishing nitrogen-fixing symbiosis with legumes: How many rhizobium recipes? *Trends in Microbiology*, *17*(10), 458–466. https://doi.org/10.1016/j.tim.2009.07.004

Maxwell, C. A., Hartwig, U. A., Joseph, C. M., & Phillips, D. A. (1989). A Chalcone and Two Related Flavonoids Released from Alfalfa Roots Induce *nod* Genes of *Rhizobium meliloti*. *Plant Physiology*, *91*(3), 842–847. https://doi.org/10.1104/pp.91.3.842

Maxwell, C. A., & Phillips, D. A. (1990). Concurrent Synthesis and Release of *nod* -Gene-Inducing Flavonoids from Alfalfa Roots. *Plant Physiology*, *93*(4), 1552–1558. https://doi.org/10.1104/pp.93.4.1552

Melino, V. J., Drew, E. A., Ballard, R. A., Reeve, W. G., Thomson, G., White, R. G., & O'Hara, G. W. (2012). Identifying abnormalities in symbiotic development between Trifolium spp. And Rhizobium leguminosarum bv. Trifolii leading to sub-optimal and ineffective nodule phenotypes. *Annals of Botany*, *110*(8), 1559–1572. https://doi.org/10.1093/aob/mcs206

Mendoza-Suárez, M. A., Geddes, B. A., Sánchez-Cañizares, C., Ramírez-González, R. H., Kirchhelle, C., Jorrin, B., & Poole, P. S. (2020). Optimizing *Rhizobium*- legume symbioses by simultaneous measurement of rhizobial competitiveness and N_2 fixation in nodules. *Proceedings of the National Academy of Sciences*, *117*(18), 9822–9831. https://doi.org/10.1073/pnas.1921225117

Mendoza-Suárez, M., Andersen, S. U., Poole, P. S., & Sánchez-Cañizares, C. (2021a). Competition, Nodule Occupancy, and Persistence of Inoculant Strains: Key Factors in the Rhizobium-Legume Symbioses. *Frontiers in Plant Science*, *12*, 690567. https://doi.org/10.3389/fpls.2021.690567

Mendoza-Suárez, M., Andersen, S. U., Poole, P. S., & Sánchez-Cañizares, C. (2021b). Competition, Nodule Occupancy, and Persistence of Inoculant Strains: Key Factors in the

Rhizobium-Legume Symbioses. *Frontiers in Plant Science*, *12*, 690567. https://doi.org/10.3389/fpls.2021.690567

Mergaert, P., Uchiumi, T., Alunni, B., Evanno, G., Cheron, A., Catrice, O., Mausset, A.-E., Barloy-Hubler, F., Galibert, F., Kondorosi, A., & Kondorosi, E. (2006). Eukaryotic control on bacterial cell cycle and differentiation in the *Rhizobium* –legume symbiosis. *Proceedings of the National Academy of Sciences*, *103*(13), 5230–5235. https://doi.org/10.1073/pnas.0600912103

Michiels, J., Dombrecht, B., Vermeiren, N., Xi, C., Luyten, E., & Vanderleyden, J. (1998). Phaseolus vulgaris is a non-selective host for nodulation. *FEMS Microbiology Ecology*, *26*(3), 193–205. https://doi.org/10.1111/j.1574-6941.1998.tb00505.x

Middleton, P. H., Jakab, J., Penmetsa, R. V., Starker, C. G., Doll, J., Kaló, P., Prabhu, R., Marsh, J. F., Mitra, R. M., Kereszt, A., Dudas, B., VandenBosch, K., Long, S. R., Cook, D. R., Kiss, G. B., & Oldroyd, G. E. D. (2007). An ERF Transcription Factor in *Medicago truncatula* That Is Essential for Nod Factor Signal Transduction. *The Plant Cell*, *19*(4), 1221–1234. https://doi.org/10.1105/tpc.106.048264

Miles, A. A., Misra, S. S., & Irwin, J. O. (1938). The estimation of the bactericidal power of the blood. *Epidemiology and Infection*, *38*(6), 732–749. https://doi.org/10.1017/S002217240001158X

Miller, L. D., Yost, C. K., Hynes, M. F., & Alexandre, G. (2007). The major chemotaxis gene cluster of *Rhizobium leguminosarum* bv. *Viciae* is essential for competitive nodulation. *Molecular Microbiology*, *63*(2), 348–362. https://doi.org/10.1111/j.1365-2958.2006.05515.x

Moawad, H., & Bohlool, B. B. (1984). Competition Among Rhizobium spp. For Nodulation of Leucaena leucocephala in Two Tropical Soilst. *APPL. ENVIRON. MICROBIOL.*, *48*.

Motnenko, A., Hawkins, J. P., Ordoñez, P. A., & Oresnik, I. J. (2025). Sinorhizobium prairiense sp. Nov., a nitrogen-fixing symbiont of Phaseolus vulgaris isolated from Canadian prairie soil. *International Journal of Systematic and Evolutionary Microbiology*, *75*(10). https://doi.org/10.1099/ijsem.0.006947

Moulin, L., Munive, A., Dreyfus, B., & Boivin-Masson, C. (2001). *Nodulation of legumes by members of the b-subclass of Proteobacteria*.

Mwenda, G. M., Hill, Y. J., O'Hara, G. W., Reeve, W. G., Howieson, J. G., & Terpolilli, J. J. (2023). Competition in the Phaseolus vulgaris-Rhizobium symbiosis and the role of resident soil rhizobia in determining the outcomes of inoculation. *Plant and Soil*, *487*(1–2), 61–77. https://doi.org/10.1007/s11104-023-05903-0

Niehaus, K., Lagares, A., & Pühler, A. (1998). A *Sinorhizobium meliloti* Lipopolysaccharide Mutant Induces Effective Nodules on the Host Plant *Medicago sativa* (Alfalfa) but Fails to Establish a Symbiosis with *Medicago truncatula*. *Molecular Plant-Microbe Interactions®*, *11*(9), 906–914. https://doi.org/10.1094/MPMI.1998.11.9.906

NIR_2023_Part1. (2023). Canadian National Inventory Report. https://publications.gc.ca/collections/collection_2025/eccc/En81-4-2023-1-eng.pdf

Onishchuk, O. P., Vorobyov, N. I., & Provorov, N. A. (2017). Nodulation competitiveness of nodule bacteria: Genetic control and adaptive significance: Review. *Applied Biochemistry and Microbiology*, *53*(2), 131–139. https://doi.org/10.1134/S0003683817020132

Oresnik, I. J., Charles, T. C., & Finan, T. M. (1994). Second site mutations specifically suppress the Fix- phenotype of Rhizobium meliloti ndvF mutations on alfalfa: Identification of a

conditional ndvF-dependent mucoid colony phenotype. *Genetics*, *136*(4), 1233–1243. https://doi.org/10.1093/genetics/136.4.1233

Oresnik, I. J., Pacarynuk, L. A., O'Brien, S. A. P., Yost, C. K., & Hynes, M. F. (1998). Plasmid-Encoded Catabolic Genes in *Rhizobium leguminosarum* bv. *Trifolii:* Evidence for a Plant-Inducible Rhamnose Locus Involved in Competition for Nodulation. *Molecular Plant-Microbe Interactions®*, *11*(12), 1175–1185. https://doi.org/10.1094/MPMI.1998.11.12.1175

Oresnik, I. J., Twelker, S., & Hynes, M. F. (1999). Cloning and Characterization of a *Rhizobium leguminosarum* Gene Encoding a Bacteriocin with Similarities to RTX Toxins. *Applied and Environmental Microbiology*, *65*(7), 2833–2840. https://doi.org/10.1128/AEM.65.7.2833-2840.1999

Page, A. J., Cummins, C. A., Hunt, M., Wong, V. K., Reuter, S., Holden, M. T. G., Fookes, M., Falush, D., Keane, J. A., & Parkhill, J. (2015). Roary: Rapid large-scale prokaryote pan genome analysis. *Bioinformatics*, *31*(22), 3691–3693. https://doi.org/10.1093/bioinformatics/btv421

Parks, D. H., Imelfort, M., Skennerton, C. T., Hugenholtz, P., & Tyson, G. W. (2015). CheckM: Assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Research*, *25*(7), 1043–1055. https://doi.org/10.1101/gr.186072.114

Pate, J. S., Layzell, D. B., & McNeil, D. L. (1979). Modeling the Transport and Utilization of Carbon and Nitrogen in a Nodulated Legume. *Plant Physiology*, *63*(4), 730–737. https://doi.org/10.1104/pp.63.4.730

Perret, X., Staehelin, C., & Broughton, W. J. (2000). Molecular Basis of Symbiotic Promiscuity. *Microbiology and Molecular Biology Reviews*, *64*(1), 180–201. https://doi.org/10.1128/MMBR.64.1.180-201.2000

Poysti, N. J., & Oresnik, I. J. (2007). Characterization of *Sinorhizobium meliloti* Triose Phosphate Isomerase Genes. *Journal of Bacteriology*, *189*(9), 3445–3451. https://doi.org/10.1128/JB.01707-06

Rai, G. K. (2019). *Developing a Rhizobial Inoculum for Phaseolus vulgaris to Increase Biological Nitrogen Fixation using Adaptive Evolution*. University of Manitoba.

Regus, J. U., Quides, K. W., O'Neill, M. R., Suzuki, R., Savory, E. A., Chang, J. H., & Sachs, J. L. (2017). Cell autonomous sanctions in legumes target ineffective rhizobia in nodules with mixed infections. *American Journal of Botany*, *104*(9), 1299–1312. https://doi.org/10.3732/ajb.1700165

Reinprecht, Y., Schram, L., Marsolais, F., Smith, T. H., Hill, B., & Pauls, K. P. (2020). Effects of Nitrogen Application on Nitrogen Fixation in Common Bean Production. *Frontiers in Plant Science*, *11*, 1172. https://doi.org/10.3389/fpls.2020.01172

Richardson, J. S., Hynes, M. F., & Oresnik, I. J. (2004). A Genetic Locus Necessary for Rhamnose Uptake and Catabolism in *Rhizobium leguminosarum* bv. Trifolii. *Journal of Bacteriology*, *186*(24), 8433–8442. https://doi.org/10.1128/JB.186.24.8433-8442.2004

Richardson, J. S., & Oresnik, I. J. (2007). L -Rhamnose Transport Is Sugar Kinase (RhaK) Dependent in *Rhizobium leguminosarum* bv. Trifolii. *Journal of Bacteriology*, *189*(23), 8437–8446. https://doi.org/10.1128/JB.01032-07

Rivers, D. M. R., Kim, D. D., & Oresnik, I. J. (2022). Inability to Catabolize Rhamnose by Sinorhizobium meliloti Rm1021 Affects Competition for Nodule Occupancy. *Microorganisms*, *10*(4), 732. https://doi.org/10.3390/microorganisms10040732

Rocha, I., Ma, Y., Souza-Alonso, P., Vosátka, M., Freitas, H., & Oliveira, R. S. (2019). Seed Coating: A Tool for Delivering Beneficial Microbes to Agricultural Crops. *Frontiers in Plant Science*, *10*, 1357. https://doi.org/10.3389/fpls.2019.01357

Rohland, N., Siedel, H., & Hofreiter, M. (2010). A rapid column-based ancient DNA extraction method for increased sample throughput. *Molecular Ecology Resources*, *10*(4), 677–683. https://doi.org/10.1111/j.1755-0998.2009.02824.x

Roslycky, E. B. (1967). *BACTERIOCIN PRODUCTION IN THE RHIZOBIA BACTERIA*.

Roughley, R. J., & Vincent, J. M. (1967). Growth and Survival of Rhizobium spp. In Peat Culture. *Journal of Applied Bacteriology*, *30*(2), 362–376. https://doi.org/10.1111/j.1365-2672.1967.tb00310.x

Rubio, L. M., & Ludden, P. W. (2008). Biosynthesis of the Iron-Molybdenum Cofactor of Nitrogenase. *Annual Review of Microbiology*, *62*(1), 93–111. https://doi.org/10.1146/annurev.micro.62.081307.162737

Salema, M. P., Parker, C. A., Kidby, D. K., & Chatel, D. L. (1982). Death of rhizobia on inoculated seed. *Soil Biology and Biochemistry*, *14*(1), 13–14. https://doi.org/10.1016/0038-0717(82)90070-0

Samago, T. Y., Anniye, E. W., & Dakora, F. D. (2018). Grain yield of common bean (Phaseolus vulgaris L.) varieties is markedly increased by rhizobial inoculation and phosphorus application in Ethiopia. *Symbiosis*, *75*(3), 245–255. https://doi.org/10.1007/s13199-017-0529-9

Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989). *v85_Ex. 1597—Sambrook 1989*. Cold Spring Harbor Laboratory Press.

Schripsema, J., De Rudder, K. E., Van Vliet, T. B., Lankhorst, P. P., De Vroom, E., Kijne, J. W., & Van Brussel, A. A. (1996). Bacteriocin small of Rhizobium leguminosarum belongs to the class of N-acyl-L-homoserine lactone molecules, known as autoinducers and as quorum sensing co-transcription factors. *Journal of Bacteriology*, *178*(2), 366–371. https://doi.org/10.1128/jb.178.2.366-371.1996

Schwinghamer, E. A. (1975). *Mic-91-2-403*. Journal of General Microbiology. https://www-microbiologyresearch-org.uml.idm.oclc.org/docserver/fulltext/micro/91/2/mic-91-2-403.pdf?expires=1774461723&id=id&accname=guest&checksum=3EBB6B96161A8AD30381ECC70DE1FCED

Schwinghamer, E. A., & Belkengren, R. P. (1968). Inhibition of rhizobia by a strain of Rhizobium trifolii: Some properties of the antibiotic and of the strain. *Archiv F□r Mikrobiologie*, *64*(2), 130–145. https://doi.org/10.1007/BF00406972

Seemann, T. (2014). Prokka: Rapid prokaryotic genome annotation. *Bioinformatics*, *30*(14), 2068–2069. https://doi.org/10.1093/bioinformatics/btu153

Shimoyama, Y. (2022). *ANIclustermap: A tool for drawing ANI clustermap between all-vs-all microbial genome* [Computer software]. https://github.com/moshi4/ANIclustermap

Simon, R., Priefer, U., & Puhler, A. (1983). A BROAD HOST RANGE MOBILIZATION SYfflM FOR IN VIVO GENRIC ENGINEERING: TRANSPOSON MUTAGENESIS IN GRAM NEGATIVE BAOERIA. *Nature*.

Spaink, H. P. (1994). The molecular basis of the host specificity of the Rhizobium bacteria. *Antonie van Leeuwenhoek*, *65*(2), 81–98. https://doi.org/10.1007/BF00871750

Sprent, J. I. (2007). Evolving ideas of legume evolution and diversity: A taxonomic perspective on the occurrence of nodulation. *New Phytologist*, *174*(1), 11–25. https://doi.org/10.1111/j.1469-8137.2007.02015.x

Sundaresan, V., Jones, J. D. G., Ow, D. W., & Ausubel, F. M. (1983). Sundaresan1983. <i> *Klebsiella Pneumoniae* <i> <i> *nifA* <i> *Product Activates the* <i> *Rhizobium Meliloti* <i> *Nitrogenase Promoter*, *301*.

Thoma, S., & Schobert, M. (2009). An improved *Escherichia coli* donor strain for diparental mating. *FEMS Microbiology Letters*, *294*(2), 127–132. https://doi.org/10.1111/j.1574-6968.2009.01556.x

Triplett, E. W. (1988). Isolation of genes involved in nodulation competitiveness from *Rhizobium leguminosarum* bv. *Trifolii* T24. *Proceedings of the National Academy of Sciences*, *85*(11), 3810–3814. https://doi.org/10.1073/pnas.85.11.3810

Triplett, E. W. (1990). Construction of a Symbiotically Effective Strain of *Rhizobium leguminosarum* bv. *Trifolii* with Increased Nodulation Competitiveness. *Applied and Environmental Microbiology*, *56*(1), 98–103. https://doi.org/10.1128/aem.56.1.98-103.1990

Triplett, E. W., & Sadowsky, M. J. (1992). *Genetics of Competition for Nodulation of Legumes*. *46*, 399–428.

Trotman, A. P., & Weaver, R. W. (1999). *Survival of rhizobia on arrowleaf clover seeds under stresses of seed-coat toxins, heat and desiccation*.

Udvardi, M., & Poole, P. S. (2013). Transport and Metabolism in Legume-Rhizobia Symbioses. *Annual Review of Plant Biology*, *64*(1), 781–805. https://doi.org/10.1146/annurev-arplant-050312-120235

Vanderlinde, E. M., Hynes, M. F., & Yost, C. K. (2014). Homoserine catabolism by *R hizobium leguminosarum* bv. *Viciae* 3841 requires a plasmid-borne gene cluster that also affects competitiveness for nodulation. *Environmental Microbiology*, *16*(1), 205–217. https://doi.org/10.1111/1462-2920.12196

Vargas, M. A. T., Mendes, I. C., & Hungria, M. (2000). Response of field-grown bean (Phaseolus vulgaris L.) to Rhizobium inoculation and nitrogen fertilization in two Cerrados soils. *Biology and Fertility of Soils*, *32*(3), 228–233. https://doi.org/10.1007/s003740000240

Vargas, M. A. T., Mendes, I. C., & Hungria, M. (2002). Response of Field Grown Bean (Phaseolus vulgaris L.) to Rhizobium Inoculation and N Fertilization in Two Cerrados Soils. In F. O. Pedrosa, M. Hungria, G. Yates, & W. E. Newton (Eds.), *Nitrogen Fixation: From Molecules to Crop Productivity* (Vol. 38, pp. 613–613). Springer Netherlands. https://doi.org/10.1007/0-306-47615-0_353

Venter, A. P., Twelker, S., Oresnik, I. J., & Hynes, M. F. (2001). Analysis of the genetic region encoding a novel rhizobiocin from *Rhizobium leguminosarum* bv. *Viciae* strain 306. *Canadian Journal of Microbiology*, *47*(6), 495–502. https://doi.org/10.1139/cjm-47-6-495

Voisin, A.-S., Prudent, M., Duc, G., & Salon, C. (2015). Pea nodule gradients explain N nutrition and limited symbiotic fixation in hypernodulating mutants. *Agronomy for Sustainable Development*, *35*(4), 1529–1540. https://doi.org/10.1007/s13593-015-0328-8

Weaver, R. W., & Frederick, L. R. (1974). *Effect of Inoculum Rate on Competitive Nodulation of Glycine maxL. tMerrill. I. GreenhouseStudies*.

Wheatley, R. M., Ford, B. L., Li, L., Aroney, S. T. N., Knights, H. E., Ledermann, R., East, A. K., Ramachandran, V. K., & Poole, P. S. (2020). Lifestyle adaptations of *Rhizobium* from rhizosphere to symbiosis. *Proceedings of the National Academy of Sciences*, *117*(38), 23823–23834. https://doi.org/10.1073/pnas.2009094117

Yates, R. J., Howieson, J. G., Reeve, W. G., & O'Hara, G. W. (2011). A re-appraisal of the biology and terminology describing rhizobial strain success in nodule occupancy of legumes in agriculture. *Plant and Soil*, *348*(1–2), 255–267. https://doi.org/10.1007/s11104-011-0971-z

Yost, C. K., Rath, A. M., Noel, T. C., & Hynes, M. F. (2006). Characterization of genes involved in erythritol catabolism in Rhizobium leguminosarum bv. Viciae. *Microbiology*, *152*(7), 2061–2074. https://doi.org/10.1099/mic.0.28938-0

Appendix 1

Presence of Plasmid-ID affects nitrogen fixation in some *Rhizobium* strains

Fluorescently tagged reporter plasmids containing unique ID regions, termed as Plasmid-IDs (M. A. Mendoza-Suárez et al., 2020) were used in this study to tag rhizobia during some of the experiments. The plasmids were constructed using the pOGG026 broad host range vector (Geddes et al., 2019) which has been shown to have apt stability in rhizobia, even in the absence of antibiotic selection markers.

The plasmid-ID backbones, which only differ in their unique ID regions, contain a consensus *nifH* promoter region that flanks a super-fold green fluorescence protein (sfGFP) coding region. This means the fluorescence would be a biomarker under the *nifH* promoter that drives the expression of the nitrogenase complex *nifHDK* (Rubio & Ludden, 2008). The consensus promoter Ps*nifH* was derived by aligning the *nifHDK* regions of 48 strains from the genus *Rhizobium*, which included an upstream activator sequence (UAS), an alternative sigma factor RpON (σ^{54}) binding site and a ribosome binding site (RBS). Together, this promoter would only be activated when the bacteria's nitrogenase promoter is activated in the presence of NifA (Sundaresan et al., 1983) which binds to the UAS and the acquisition of the alternat sigma factor (RpON). According to the developers of the plasmid-IDs, the fluorescent bioreporter should reflect the expression of *nifH* as well as the bacteroid population, which would directly correlate to the effectiveness of nitrogen fixation (Melino et al., 2012; Regus et al., 2017). This means, the intensity of the glow of the nodules would correlate to the effectiveness of nitrogen fixation of the plasmid tagged rhizobia in the nodules.

During the preliminary phases of the thesis, it was noticed that the presence of plasmid-IDs was affecting the nitrogen fixation of some strains of rhizobia that were being tested. To

Table 8: List of *Rhizobium* strains and plasmids (Appendix 1)

Strain/plasmid	Species/plasmid description	Reference or point of isolation
UMR103	*R. hidalgonese*	49.497316, -98.045345
UMR106	*R. leguminosarum*	49.497316,-98.045345
UMR330	*R. croatiense*	49.926764, -98.531525
pOPS0523(C9)	psNifH-sfGFP-C9 in pOGG026 Plasmid constructed by GG with universal primer binding site. Confirmed ID sequence: GCATATGCACTG Golay reference name:806rcbc32. Nmr/Kanr	(M. A. Mendoza-Suárez et al., 2020)
pOPS0526(C12)	psNifH-sfGFP-C12 in pOGG026 Plasmid constructed by GG with universal primer binding site. Confirmed ID sequence: TACGAGCCCTAA Golay reference name:806rcbc35. Nmr/Kanr	(M. A. Mendoza-Suárez et al., 2020)

Figure 16: Plants inoculated with UMR106 and the plasmid-ID tagged variant
Individual navy bean seedlings were inoculated with 10^8 cells of UMR 106 (left) and UMR106+ pOPS0523 (Right) and harvested 28 days after inoculation. Preliminary visual inspection of the plants shows insufficient nitrogen fixation in the tagged variant plants deemed by the yellow leaves and the brittle appearance of the plants.

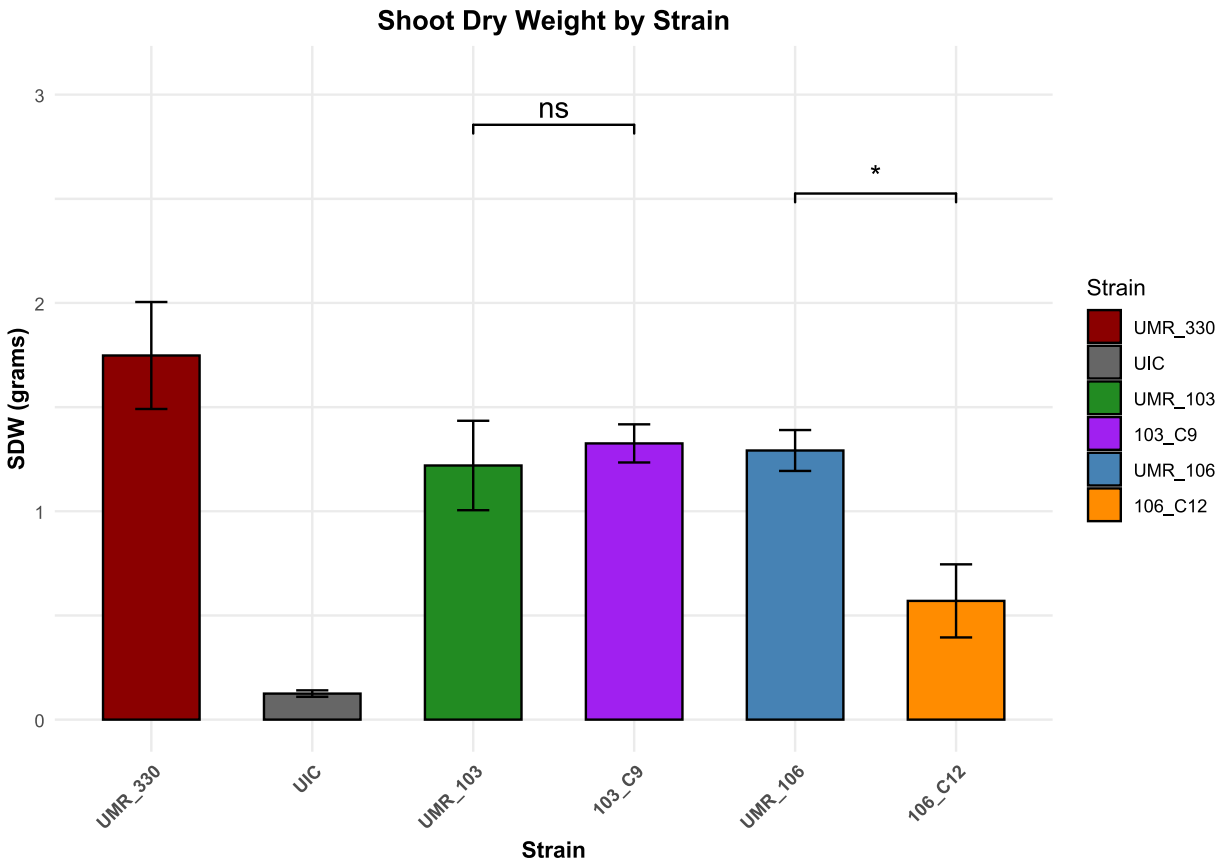

Figure 17: Shoot dry-weight of plants inoculated with UMR strains and the plasmid-ID tagged variants.

Individual navy bean seedlings were inoculated with wild-type UMR strains and their tagged variants. UMR 330 was used as positive control, and UIC denotes un-inoculated plants as a negative control. The X-axis represents strains, and the Y-axis represents dry mass in grams. Dry mass of UMR103 and its tagged variant shows no significant difference, however the dry mass of UMR106 and its tagged variant shows significant decrease. A t-test was used to measure significance. (*) denoted p-value < 0.05.

All measurements are the average of 4 replicates, and the error bars denote standard error.

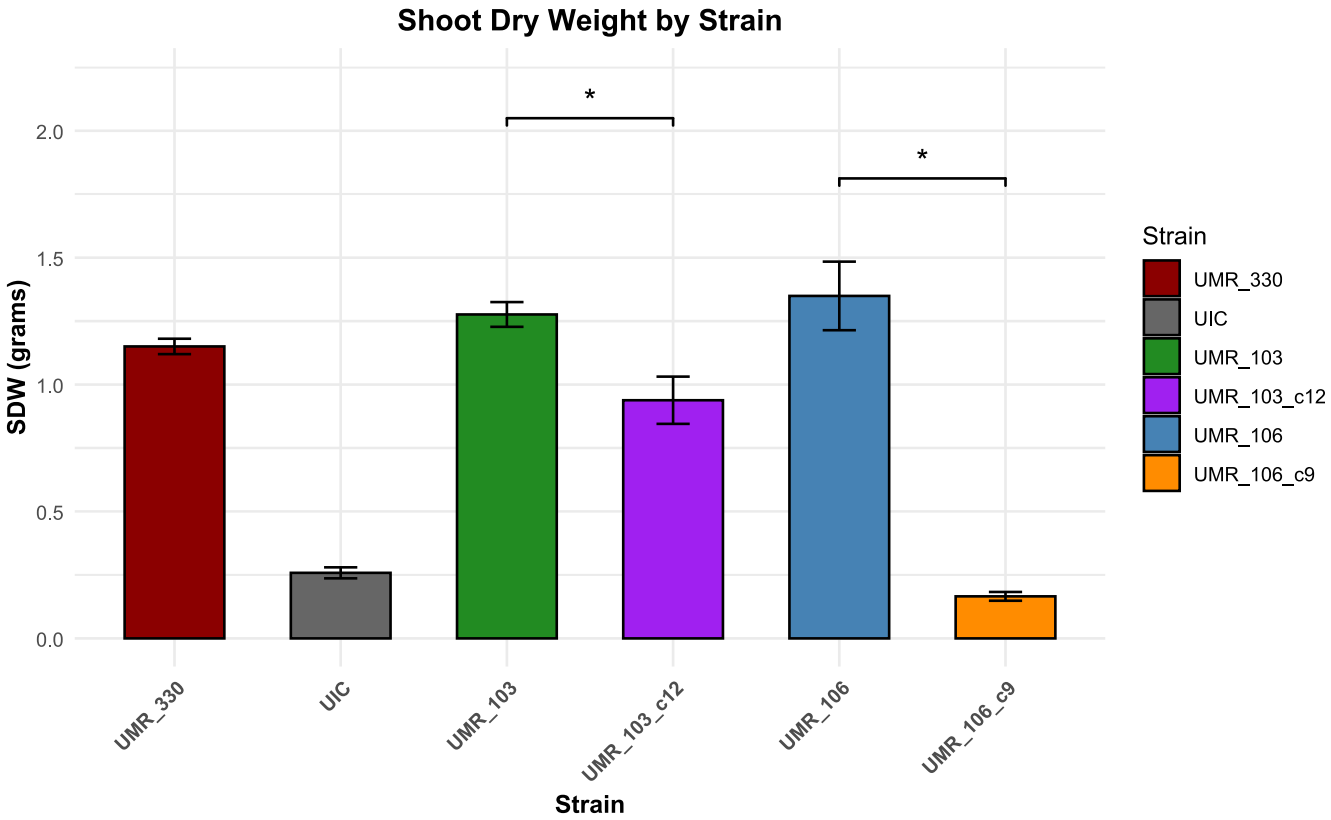

Figure 18: Shoot dry-weight of plants inoculated with UMR strains and the tagged variants with the plasmid-IDs interchanged

Individual navy bean seedlings were inoculated with wild-type UMR strains and their tagged variants. The plasmid-IDs were flipped between the wild-type strains. UMR 330 was used as positive control, and UIC denotes un-inoculated plants as a negative control. The X-axis represents strains, and the Y-axis represents dry mass in grams. Dry mass of UMR103 and its tagged as well as the dry mass of UMR106 and its tagged variant shows significant decrease. A t-test was used to measure significance. (*) denoted p-value < 0.05.

All measurements are the average of 4 replicates, and the error bars denote standard error.

empirically test the hypothesis, two strains of rhizobia, isolated from Manitoban soil, and two plasmid-IDs were used (Table 8). To create tagged version of the wild-type strains, two plasmid-IDs were used, where the backbone of both plasmids is the same and they only differ in their synthetic 12 nucleotide ID regions.

Both wild-type strains (UMR103 and UMR106) and their corresponding tagged versions (UMR103+ pOPS0523 and UMR106+ pOPS0526) were used to inoculate navy bean seedlings grown in nitrogen-free nutrient medium. This was done in 4 biological replicates. The plants were harvested 28 days after inoculation, the shoots were dried to measure the shoot dry-weight (SDW) and their roots were visualized for fluorescence under blue light. The mass of the dry matter would correlate to the ability of fixed nitrogen, and the glow of the nodules of the tagged strains should corroborate that. This was done with the plasmids switched between either strain, meaning two more transconjugants were constructed (UMR103+ pOPS0526, and UMR106+ pOPS0523). The inversions were done to rule out any defect of any specific plasmid backbone, which should be the same for both plasmids.

In all cases, the wild-type strains showed higher dry-mass compared to their tagged variants. Moreover, one strain in specific, UMR 106 displayed significant attenuated nitrogen fixation when tagged with either plasmid-IDs. Plants inoculated with tagged variants of UMR106 displayed a sickly yellow appearance (Figure 16), indicating insufficient nitrogen fixation. The dry mass of those plants was also noticed to be significantly reduced compared to their wild-type counterpart (Figure 17). This was also true when the plasmid-IDs were switched between the two strains (Figure 18). When the nitrogenase activity was measured using a hydrogen analyzer, the tagged UMR106 variants showed significantly reduced nitrogenase activity (Figure 19). Using a

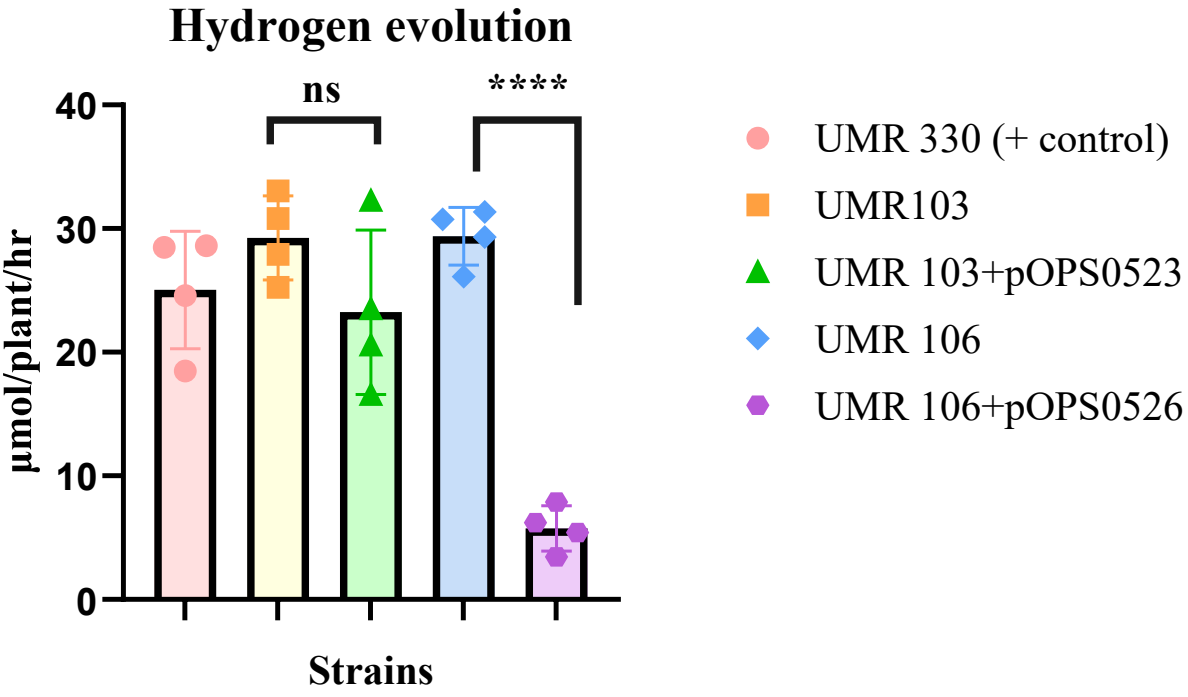

Figure 19: Hydrogen evolution of the whole root system of plants inoculated with wild-type UMR strains and the plasmid-ID tagged variants.
Individual navy bean seedlings were inoculated with 10^8 cells and harvested after 28 days after inoculation. The plants were uprooted and their nitrogenase activity of the whole root system containing all nodules was measured using a hydrogen analyzer. The X-axis contains the strains used for the experiment and the Y-axis represents µmol of hydrogen evolved per plant per hour. This is a proxy measurement for fixed nitrogen.
UMR 106 shows significant reduction in nitrogenase activity (p value < 0.0001) while UMR103 shows no significant reduction in nitrogenase activity. A standard two-tailed t-test was used to measure significance.

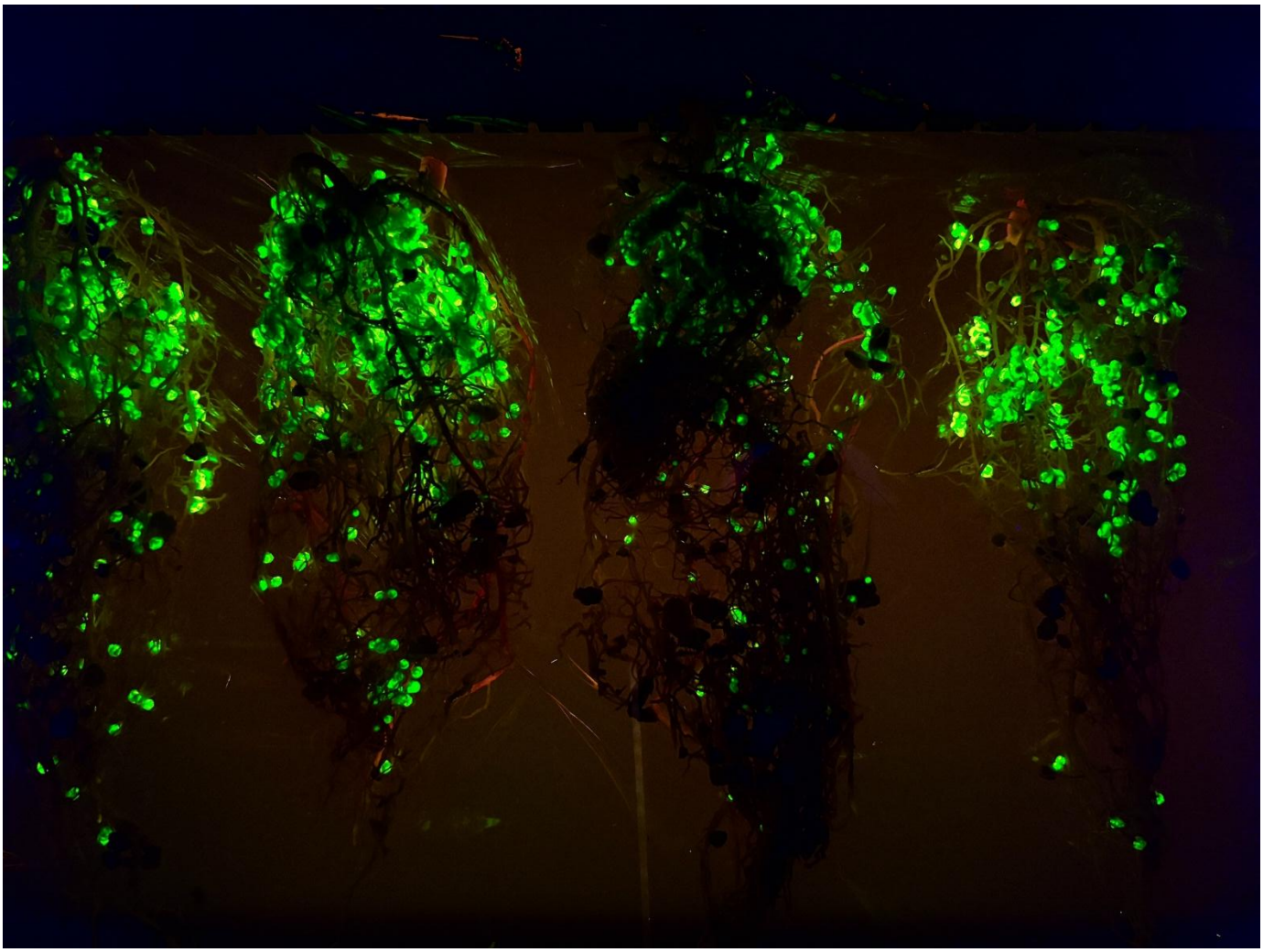

Figure 20: Nodules of UMR106+ pOPS0526 visualized using blue light.
Plants inoculated with UMR106+ pOPS0526 were grown for 28 days and the roots were washed with sterile water and visualized under blue light. The image shows robust activation of the sfGFP under the nitrogenase promoter in the plasmid-ID, resulting in bright green glowing nodules.

two-tailed t-test, a significant reduction ($p < 0.0001$) in nitrogenase activity of UMR106 in the presence of the plasmid was noticed, whereas no significant difference was noticed in UMR103. Interestingly, when the roots of the low dry mass and attenuated nitrogenase plants containing tagged UMR106 variants were visualized using blue light, the nodules glowed extremely brightly (Figure 20). This conflicts both the other measurements taken in this study, as well as the claim of the original developers of the plasmid that the fluorescence would correlate to nitrogenase activity. The same was not noticed for UMR 103. Though the shoot dry-mass of the tagged variants of UMR103 were slightly less compared to its wild-type counterpart, the nitrogenase activity was not severely attenuated and the glow of the nodules did not show contrary evidence of nitrogenase attenuation. The plants of the tagged variants of UMR103 were healthy, displayed glowing nodules and fair nitrogenase activity.

These observations further support the hypothesis that the presence of plasmid-IDs affects some *Rhizobium* strains. Due to limitations in time, further characterization as to why this happens could not be done using standard molecular and genetic techniques. However, the observations could be used in the future to note the construction and validation of biomarkers placed under the nitrogenase promoter, as well as genetic regulations of the NifA-RpON system in rhizobia.

Appendix 2

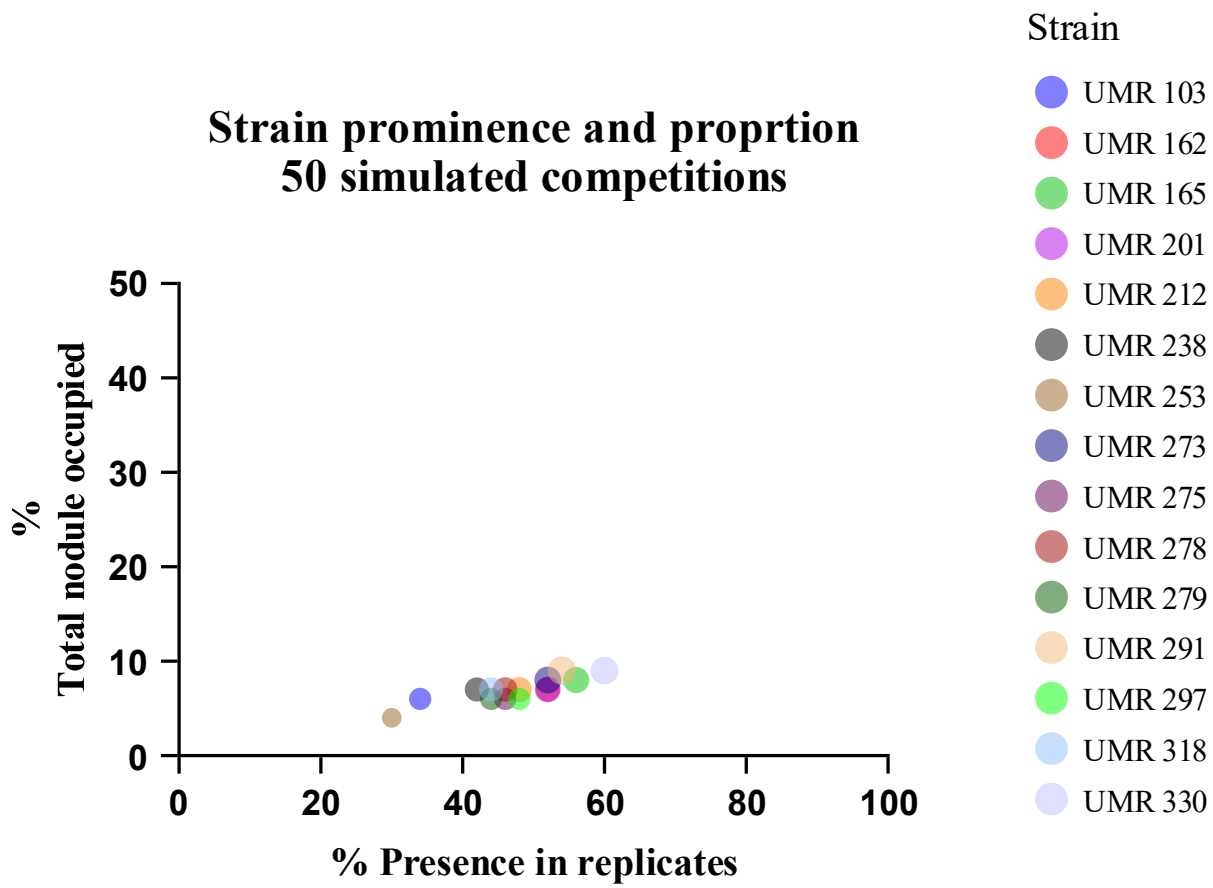

Figure 21: Strain prominence and proportion of strains in simulated competition events.
Strain prominence and proportion were calculated in an artificially simulated competition experiment with the parameters that all strains have the same competitive advantage and selection of nodules are random.
A simulation was done where the iterative process of the multi-iterative competition screening was emulated and the results of the final iteration were noted. This was done 50 separate times and the results of the final iteration of all 50 were used to calculate the prominence and proportion of the strains. The average proportion of nodules was 7%±1% and the prominence was 47%±8%.