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Exploring the ecological and evolutionary impacts of introducing exotic agricultural inoculants to native soil microbiomes

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Author's declaration

I, Grace Ella Wardell, confirm that this thesis is my own work. I am entirely aware of the guidance of the University on the Practice of Unfair Means (www.sheffield.ac.uk/ssid/unfair-means). This work has never been previously presented for an award at this, or any other degree or any other university.

Thesis Summary

Microbial inoculants offer a more sustainable approach to providing crops with essential nutrients than conventional inorganic fertilisers, however the ecological and evolutionary impacts of introducing inoculants to soil microbiomes are not well understood. One group of bacteria, collectively known as rhizobia, enter into symbiotic interactions with legumes and fix atmospheric N_2 into plant-available nitrogen in exchange for carbon compounds. Inoculating legumes with compatible rhizobia can result in biological nitrogen fixation, however, in an environment where compatible rhizobia are absent from the soil microbiome, introduction may have various impacts on the receiving microbial community. The relatively recent expansion of soybean (*Glycine max* L. Merr) growth in UK agriculture, where seed inoculation with exotic rhizobia is common, offers an opportunity to study the impact of introducing non-native rhizobia to soil microbiomes. Understanding interactions between inoculant and resident communities could improve the efficacy of soybean inoculant products, leading to increased crop yields and soybean production in the UK. Utilising a combination of greenhouse experiments, fieldwork and bioinformatics approaches, this thesis explores the ecological and evolutionary impacts of introducing soya-nodulating rhizobia (SNR) to UK soil communities. Further, the impacts of inoculating a diverse consortium, with multiple compatible rhizobia species or in combination with plant growth-promoting rhizobacteria (PGPR) on temperate-adapted soybean plant productivity was explored. Results highlighted that inoculation with non-native rhizobia altered soil bacterial community dynamics transiently, however the inoculant strains persist within the community. Investigating multi-species rhizobia inoculants for temperate soybean showed that *Bradyrhizobium* symbionts performed better than *Sinorhizobium* symbionts and co-inoculation with PGPR uncovered a beneficial association between *Bradyrhizobium* inoculants and *Rhizobium languerre* PEPV16. Finally, evidence of inoculant evolution was discovered during the first season of soybean and SNR introduction into UK agricultural field

sites. This research contributes to our understanding of inoculation impact for their safer utilisation in cropping systems.

Table of Contents

List of Figures

Chapter 1

Chapter 2

Supplementary Figures

Chapter 3

Chapter 4

Chapter 5

List of Tables

Chapter 2

Supplementary Tables

Chapter 3

Chapter 4

Supplementary Tables

Chapter 5

Chapter 1: Introduction

1.1 Microbial inoculants for more sustainable agriculture

The soil microbiome is one of the most diverse environments on Earth, where inhabiting microorganisms possess a range of beneficial functions that can aid plant growth and contribute to ecosystem functioning (Wagg *et al.*, 2014). One specialised polyphyletic group of bacteria, collectively known as rhizobia, converts atmospheric nitrogen (N_2) into nitrogenous compounds that legume (Fabaceae) plants can utilise, in exchange for carbon compounds in a tightly regulated symbiosis that is of global economic and ecological importance (Sprent, Ardley and James, 2017). Inoculating legume seeds and/ or soil with rhizobia can increase or introduce biological nitrogen fixation (BNF) to an area, potentially reducing the need to apply nitrogen (N) fertilisers to legume crops (Araujo, Urbano and González-Andrés, 2020). The application of N fertilisers in agriculture is one of the biggest causes of nitrous oxide (N_2O) greenhouse gas emissions globally (Liu, Guo and Xiao, 2019) and can result in pollution of other environments via N losses from agricultural systems (Robertson and Vitousek, 2009). For this reason, inoculating crops with beneficial microorganisms is a more sustainable agricultural practice which can reduce detrimental environmental consequences (Araujo, Urbano and González-Andrés, 2020; Mendoza Beltran *et al.*, 2021). Rhizobia are chemotactically attracted to legume flavonoids exuded from roots, and in response, they produce lipochitooligosaccharides known as nodulation (nod) factors (Long, 2001). Both of these plant- and bacteria-associated signals, along with other genetic factors, contribute to the compatibility of the symbiotic relationship (Roche *et al.*, 1996; Liu and Murray, 2016; Wang, Liu and Zhu, 2018). If compatible rhizobia are present in a soil microbiome, legumes will form specialised root structures called nodules, which house the bacteria as they differentiate into cells capable of expressing the nitrogenase enzyme (now termed bacteroids), which is essential for performing symbiotic BNF (Poole, Ramachandran and Terpolilli, 2018). The symbiosis is tightly regulated, with the

legume host able to impose multiple compatibility checkpoints (Zgadzaj *et al.*, 2015; Kusakabe *et al.*, 2020) and sanction nodules that are inefficient at providing N (Kiers *et al.*, 2003; Westhoek *et al.*, 2017, 2021), reducing the potential for nodules to be colonised by "cheating" rhizobia. Some legume- rhizobia symbioses can be promiscuous, with hosts able to enter interactions with a range of rhizobial symbionts (Shamseldin and Velázquez, 2020), whereas others, such as soybean (*Glycine max* L. Merr), require specific partners (Bellato *et al.*, 1997; Wang, Liu and Zhu, 2018). Although inoculation is not always essential when growing legumes in a native or now naturalised range (Maluk *et al.*, 2022), a high degree of partner specificity means that when introducing a legume crop to a new area, inoculation with compatible rhizobia is essential to result in nodulation and efficient BNF (Parker, 2001; Le Roux *et al.*, 2017; Maluk *et al.*, 2023). However, little research has investigated the impact of inoculating non-native rhizobia into indigenous soil microbiomes.

1.2 Ecological and evolutionary impacts of rhizobia inoculation

Inoculation requires introducing a large population of bacteria to the soil microbiome (~1 billion colony forming units per seed), which, when scaled up to an agricultural field, becomes a mass species introduction. There can be ecological and evolutionary impacts of microbial introduction, which can form eco-evolutionary feedback loops (terHorst and Zee, 2016). For example, inoculation can lead to transient or long-lasting perturbations on soil microbial communities, which may have knock-on effects for the functional diversity of those communities (Trabelsi and Mhamdi, 2013; Ambrosini, de Souza and Passaglia, 2016; Mawarda *et al.*, 2020). Even inoculations that result in unsuccessful bacterial colonisation can alter the microbial communities niche structure (Mallon *et al.*, 2018). With regards to non- native legume introduction to a new environment, inoculation with compatible rhizobia adds a novel niche to the soil microbiome, nodulation and BNF with the novel legume species. Introducing compatible rhizobia alongside non-native legumes provides the introduced rhizobia with a specific niche in an otherwise competitive, locally adapted soil

microbial community, which can contribute to their successful establishment (Litchman, 2010; Ambrosini, de Souza and Passaglia, 2016). Repeated growth of host legume species can boost symbiont populations as rhizobia can proliferate a million fold within nodules (Denison and Kiers, 2011), with large populations returned to the soil microbiome, forming positive plant-soil feedback loops. The introduction of exotic inoculant strains also introduces novel genetic material to the microbial community. Rhizobia have diverse lifestyles, as soil-dwelling saprophytes and as plant-associated endosymbionts (Poole, Ramachandran and Terpolilli, 2018). This is reflected in their adaptable genomes, where the symbiosis genes are harboured on mobilisable DNA, including accessory plasmids or integrative conjugative elements (ICE); regions of DNA that can integrate into and excise from bacterial chromosomes (Maclean, Finan and Sadowsky, 2007; Ding and Hynes, 2009; Haskett *et al.*, 2016; Colombi *et al.*, 2021; Weisberg *et al.*, 2022). Horizontal gene transfer (HGT) of the symbiosis genes can occur, resulting in inoculant symbiosis genes entering native rhizobia genetic backgrounds (Sullivan *et al.*, 1995; Barcellos *et al.*, 2007; Hill *et al.*, 2021). Additionally, inoculant strains can evolve due to the nature of inoculant production, strains are grown to high population densities in a nutrient rich environment followed by different selection pressures when introduced to novel soil environments (Takors, 2012; Kaminsky *et al.*, 2019). Varying outcomes of these evolutionary processes may lead to different outcomes on the symbiosis, for example, the creation of locally adapted symbionts with superior or similar nodulation and N_2 fixation abilities as the inoculant strains (Batista *et al.*, 2007; Hill *et al.*, 2021; Colombi *et al.*, 2023), ranging to symbionts that can nodulate the host, but are ineffective at N_2 fixation (Nandasena *et al.*, 2006, 2007), resulting in suboptimal crop yields. Thus, the ecological and evolutionary effects of inoculation can have substantial impacts on important legume crop yields, warranting further investigation into interactions between inoculants and native populations.

1.3 Soybean production in the UK

The introduction of soybean into UK agriculture provides a good opportunity to study the impacts of inoculating exotic rhizobia into a microbiome that lacks this function (soya-symbiosis). As one of the most globally valuable crops, soybean demand is predicted to increase and is now becoming a commercial reality in the UK, with around 10,000 ha grown currently (UK Roundtable and EFECA, 2018; Soya UK, 2019; Coleman *et al.*, 2021; NIAB, 2021). Originating from East Asia, soybean and its rhizobia partners have now been co-introduced to large parts of the world, with the largest production areas today being Brazil, USA and Argentina (Thilakarathna and Raizada, 2017; Santos, Nogueira and Hungria, 2019). Soybean's predominant rhizobial symbionts are from the genera *Bradyrhizobium* and *Sinorhizobium* (formerly *Ensifer),* but current commercial soybean inoculants only contain *Bradyrhizobium* species (Hungria *et al.*, 2006; Santos, Nogueira and Hungria, 2019). There is an opportunity to explore the potential use of *Sinorhizobium* symbionts as inoculants due to their adaptation to more alkaline soils (Tian *et al.*, 2012), which overlap with soils within the suggested soya suitability range in the UK (Figure 1). UK soils possess native *Bradyrhizobium* species that nodulate native legumes, such as gorse (*Ulex europeaus* L.) and broom (*Cytisus scoparius* L.) (Rogel, Ormeño-Orrillo and Martinez Romero, 2011; Sprent, Ardley and James, 2017; Stępkowski *et al.*, 2018) and *Sinorhizobium* species that nodulate Lucerne/Alfalfa (Medicago sativa L.), other Medics and *Melilotus* species (Roberts *et al.*, 2017). However, these are incompatible as soybean symbionts. As soybean growth and inoculation is still in its infancy in the UK, there is an opportunity to improve inoculant efficacy by investigating the potential for inoculating a diverse consortia containing multiple compatible rhizobia (Fields *et al.*, 2021), and the combination with other beneficial plant growth-promoting rhizobacteria (PGPR) to improve plant yields (Zeffa *et al.*, 2020). Research in this area can have valuable contributions to the applicability of inoculant products.

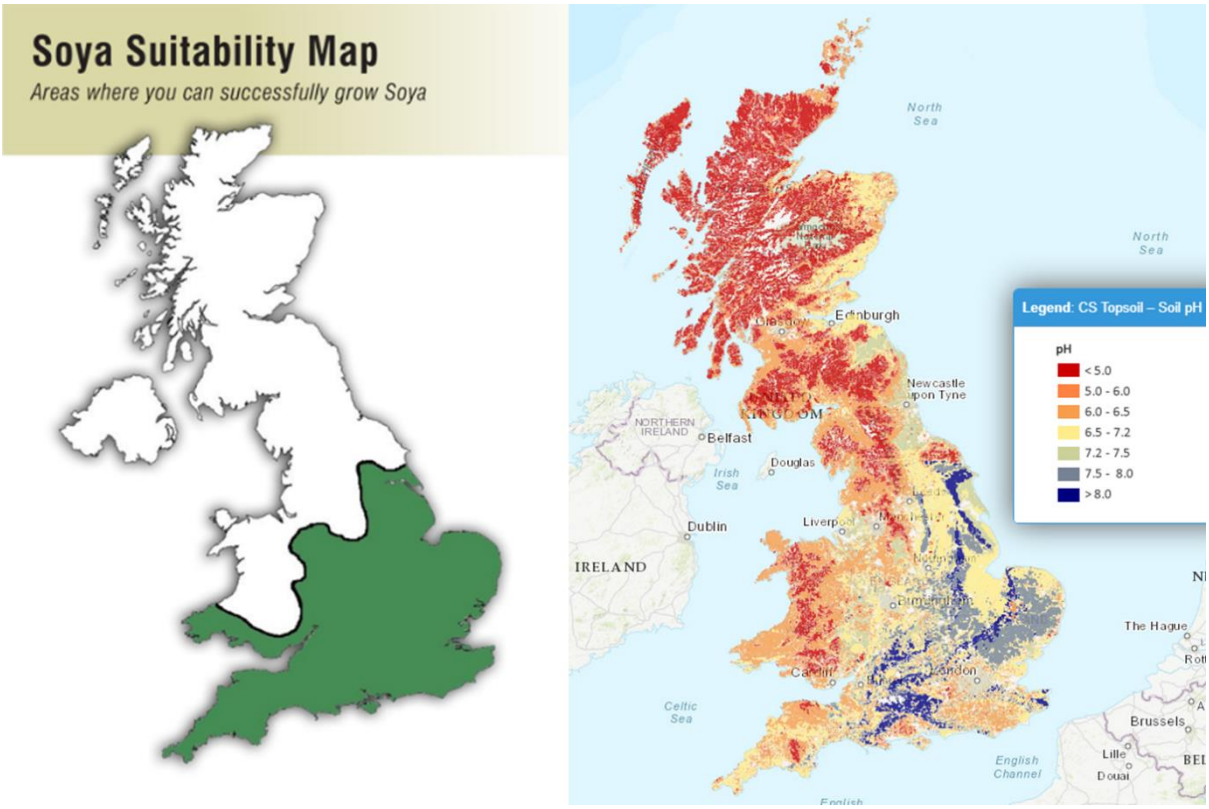

Figure 1. Left; suggested suitability map for growing soybean in the UK (Soya UK, 2019). Right; topsoil pH across UK soils (UKSO, 2023).

1.4 Rationale and Overview

The introduction of soya-nodulating rhizobia (SNR) to the UK soil microbiome could lead to improved soybean crop yields with reduced N fertiliser inputs, but the potential impacts on the soil microbiome are not well understood. Additionally, improving inoculant formulations, either through the addition of multiple compatible rhizobia or PGPR could lead to an improvement in crop yields for this underutilised, emerging crop in the UK (DEFRA, 2022). The research presented in this thesis aims to increase our knowledge about inoculum impact on native microbial communities and the potential for inoculant improvement for soybean in the UK. Figure 2 is a schematic overview of the themes investigated in this thesis, where the numbers correspond to the following sections:

1. *Chapter 2* investigates the ecological impacts of exotic rhizobia inoculation on the native soil microbial community, and by extension the native rhizobia community.

2. *Chapter 3* follows up findings from Chapter 2 investigating the impact of multiple compatible rhizobia inoculant strains on soybean plant biomass yields.
3. *Chapter 4* assesses the combination of rhizobia and PGPR inoculation on soybean biomass for potential future inocula formulations.
4. *Chapter 5* explores inoculant evolution in the first season of SNR introduction to UK farm sites.
5. Appendix: 'Why are rhizobial symbiosis genes mobile?' (Phil Trans B) review delves into the widespread occurrence of symbiosis element HGT and its impact on rhizobial ecology and evolution.

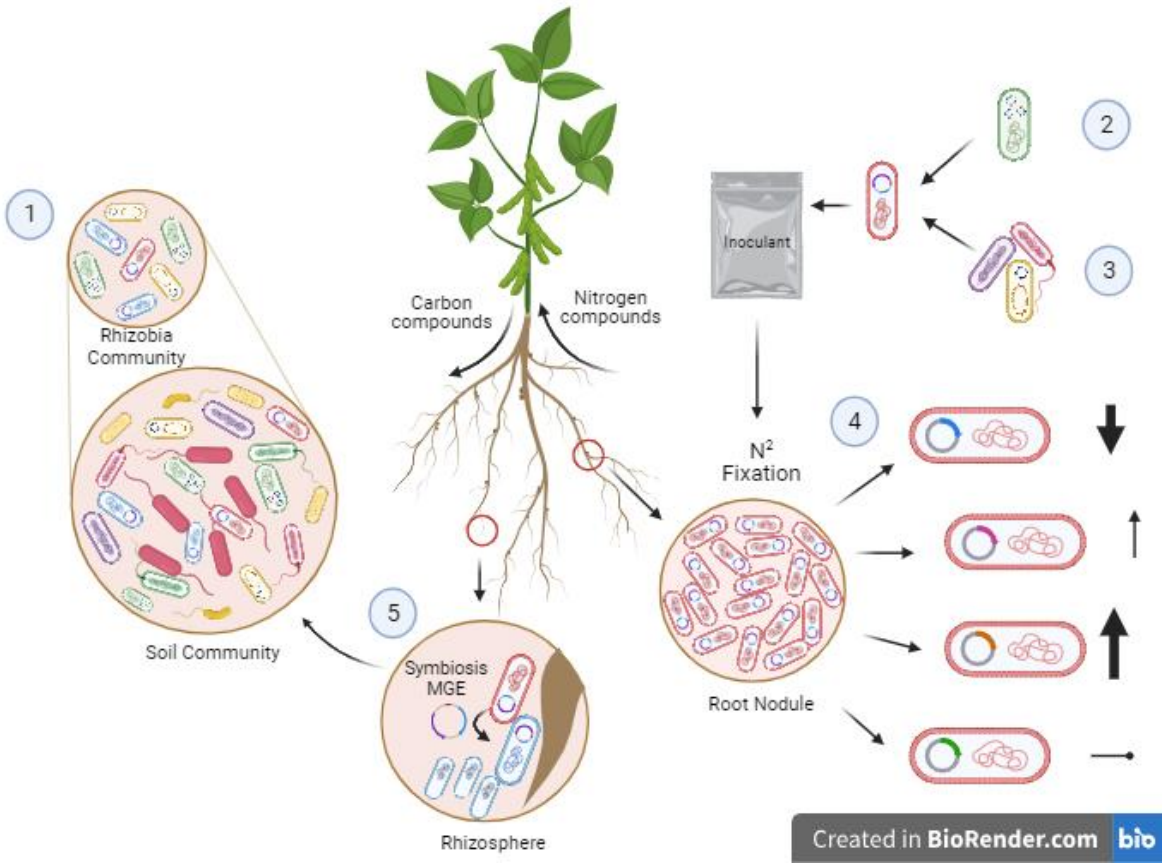

Figure 2. Schematic overview of the themes explored in this thesis. Created with Biorender.

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Chapter 2: Inoculation of exotic soybean rhizobia induces transient impacts on soil communities, but biological nitrogen fixation persists

Aimed at Environmental Microbiome

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2.1 Background: Inoculating legumes with compatible rhizobia is common practice in agriculture. The growth of novel legumes outside of their native range necessitates inoculation with non-native rhizobia to provide biological N_2 fixation, reducing the need for N fertilisers. However, there is a gap in research on how these exotic inoculants may affect the soil microbiome, including how it impacts soil bacterial community dynamics and more specifically, the indigenous rhizobial community. Soybean production is increasing in the UK yet compatible symbiotic partners are absent from the soil microbiome, offering an opportunity to study the effects of exotic bacterial inoculation. This study examines the impact of soybean inoculation in two soils, with and without a history of exposure to soybean inoculants. Two different rhizobia species were used as inoculants, including one previously introduced in the field, *Bradyrhizobium diazoefficiens,* and one novel, *Sinorhizobium fredii*.

Results: Using amplicon sequencing and qPCR to track the inoculant strains in the microbiome, high persistence of the original inoculant strains was observed; which provided yield benefits to a temperate soybean variety, but negated the impacts of new inoculation. Inoculation resulted in transient effects on soil communities throughout the soybean growing period. An inoculant species-specific effect on the indigenous rhizobia community, was observed with *B. diazoefficiens* inoculation significantly affecting *Alphaproteobacteria* communities two days post inoculation. Inoculation altered total bacterial community dynamics during peak nitrogen fixation, however soil type and temporal effects were greater.

Conclusions: Inoculation results in transient effects on soil bacterial communities, however the inoculants and function (soya- symbiosis) persists in the microbiome. Understanding interactions between non-native inoculants and indigenous microbial communities can inform the development of more efficient microbial inoculant technologies, for their safer utilisation for more-sustainable cropping systems.

Keywords: soybean, rhizobia, inoculants, soil, microbiome.

2.2 Background

Microbial inoculants are emerging as a key strategy for sustainable agriculture and N_2 fixing inoculants make up 80% of the global biofertiliser market (Basu *et al.*, 2021). Inoculation of legume crops with their N_2- fixing symbionts (rhizobia) is a long-standing practice (Santos, Nogueira and Hungria, 2019). When introducing a legume crop to a new range, inoculation with compatible rhizobia is necessary for the establishment of a root-nodulating symbiosis capable of biological nitrogen fixation (BNF), minimising the need for N fertilisers and ensuring optimal yields. In recent years, temperate adapted soybean (*Glycine max* L. Merr) varieties are being grown at higher latitudes and their compatible rhizobial symbionts introduced (Kühling *et al.*, 2018). Soybean is currently shortlisted as an underutilised crop in the UK (DEFRA, 2022). The UK imports an estimated 3.8 million tonnes of soya annually (UK Roundtable and EFECA, 2018), but the development of early maturing varieties (Zimmer *et al.*, 2016) and changing climatic conditions means soya cultivation in the UK is predicted to increase in suitable agricultural areas (Coleman *et al.*, 2021), which could reduce reliance on imports. Evidence from the widespread introduction of soybean to continents outside its native East Asian origin has highlighted the need to co-introduce compatible symbionts; since the indigenous rhizobial communities cannot nodulate soybean (Mendes, Hungria and Vargas, 2004; Satya Prakash and Annapurna, 2006; Zimmer *et al.*, 2016; Kühling *et al.*, 2018; Maluk *et al.*, 2023). The main soybean rhizobial symbionts reside within the alphaproteobacterial genera *Bradyrhizobium* and *Sinorhizobium,* of which UK soils naturally possess native *Bradyrhizobium* and *Sinorhizobium* species, but these do not possess the symbiosis genes required to nodulate soybean (Rogel, Ormeño-Orrillo and Martinez Romero, 2011; Roberts *et al.*, 2017; Sprent, Ardley and James, 2017; Stępkowski *et al.*, 2018; Maluk *et al.*, 2023). Most soybean inoculants globally (and in the UK) are composed of two *Bradyrhizobium* strains that enable good crop performance (so called ‘elite’ strains), *Bradyrhizobium japonicum* SEMIA 5079 and *Bradyrhizobium diazoefficiens* SEMIA

5080, and are the result of rhizobial strain selection programmes from the World-leading inoculation industry in Brazil (Hungria and Vargas, 2000; Siqueira *et al.*, 2014; Santos, Nogueira and Hungria, 2019).

Introduction of a large population of bacteria can alter the native microbiome's community composition and diversity (Zhang *et al.*, 2011; Trabelsi and Mhamdi, 2013; Ambrosini, de Souza and Passaglia, 2016; Zhong *et al.*, 2019; Mawarda *et al.*, 2020; Xu *et al.*, 2020). For inoculant strains to efficiently establish and supply a beneficial function to the soil microbiome, they are applied in large doses. For example, inoculation with SNR in Brazil requires use of liquid inoculant concentrations of 1 x 10^9 viable cells per seed (Santos, Nogueira and Hungria, 2019). Recipient microbiomes can either be resistant to the introduced inoculant, remaining unchanged, or experience transient or lasting impacts on community structure (Mawarda *et al.*, 2020). Ecological mechanisms such as resource competition, synergistic or antagonistic interactions between inoculant and resident populations and indirect effects such as stimulation of plant root exudates, can result in changes in community composition which can have knock-on effects on the function of that microbiome (Bell *et al.*, 2005; Mawarda *et al.*, 2020). In addition, microorganisms that possess an affinity for certain plants and strongly affect plant growth, such as host specific rhizobia, are more likely to impact plant-soil feedbacks (terHorst and Zee, 2016) and thus may have a greater impact on the recipient soil microbiome than the addition of free-living PGPRs (Ambrosini, de Souza and Passaglia, 2016). The coupled introduction of a protective niche (root-nodules) alongside inoculant rhizobia can be advantageous for establishment, when such inoculants are introduced to an already diverse microbial community with limited niche availability and high resource competition (Mallon *et al.*, 2015). Although there is some research investigating the impacts of rhizobial inoculation on soil communities, this has primarily been conducted either in regions where the host and compatible symbionts are native, e.g. in China for soybean (Zhang *et al.*, 2011; Zhong *et al.*, 2019; Xu *et al.*, 2020) or where symbiont populations have been introduced and have become naturalised e.g., in the

Americas (Souza *et al.*, 2016; Leggett *et al.*, 2017; Bender *et al.*, 2022). No research has focused on assessing impacts of exotic rhizobial inoculation on microbial communities in non-native areas.

The introduction of exotic, elite inoculant strains may affect the indigenous rhizobial community. Inoculation with rhizobia can increase (Trabelsi *et al.*, 2011), or decrease (Zhang *et al.*, 2010) the diversity of this functional group. Novel bacteria can also introduce novel traits, in this case, the soya specific symbiosis genes, which can be readily mobilised as they are harboured on mobile elements (Remigi *et al.*, 2016; Wardell *et al.*, 2022; Weisberg *et al.*, 2022). Such transfer events are more likely to occur between closely related strains (Andrews *et al.*, 2018). Transfer of novel soya-specific symbiosis genes into native communities has been observed in other non-native regions of soya production (Satya Prakash and Annapurna, 2006; Barcellos *et al.*, 2007; Batista *et al.*, 2007). Transfer of this trait can lead to a pool of symbionts with varying symbiotic capabilities (Nandasena *et al.*, 2006, 2007; Heath *et al.*, 2022; Weisberg *et al.*, 2022), which can feedback into ecological processes between symbiont populations (Rahman *et al.*, 2023), such as increased competition for resources in the soil microbiome or plant colonisation. Understanding interactions between inoculant strains and related communities in particular may therefore serve to better predict potential eco-evolutionary feedbacks in the long term. Additionally, the impacts of inoculation on a microbial community may differ depending on whether it has received the exotic rhizobia before. Often rhizobia inoculants are found to remain in the soil microbiome after the season it has been inoculated (Narozna *et al.*, 2015; Giongo *et al.*, 2020). This persistence can sometimes affect the impacts of (re)-inoculation (Ambrosini *et al.*, 2019; Halwani *et al.*, 2021; Zilli *et al.*, 2021).

Here we investigate the impact of inoculating soybean with exotic rhizobia on soil microbiomes from different fields on a single UK farm; one field having no history of soybean cultivation or inoculation and the second with previous cultivation of soybean including

inoculation with a commercial inoculant (containing *B. japonicum* SEMIA 5079 and *B. diazoefficiens* SEMIA 5080). In a mesocosm experiment, we inoculated soybean with a single *Bradyrhizobium diazoefficiens* strain, one of the inoculant strains widely used across the UK, or with a *Sinorhizobium fredii* strain, representing a soybean inoculant entirely novel to the UK. We report on the abundance of inoculant strains in the soil microbiome and monitor community composition and diversity of total bacterial- and rhizobial-populations throughout the soybean growth period.

2.3 Methods

Soil sampling

Agricultural topsoil for the experiment was sampled in December 2019 from two agricultural fields on a single farm in Kent, UK. Field 1 (51.31 lat, 0.76 long) had no previous history of soybean crop and its associated rhizobia (hereon defined as no-soya soil), while field 2 (51.32 lat, 0.77 long) had seed inoculated (Legume Technology, East Bridgford, UK) soybean (cv. Siverka, SoyaUK, Hampshire, UK) grown in the spring/summer growth season of 2019 (hereon defined as soya soil; Table S1). This was the third year that soybean had been grown and inoculated on this farm, but all in different fields. Soils were sampled at least 10 m from the margin of the fields, along a 500 m transect, six 10 L topsoil samples were taken to a depth of 20 cm. Due to the COVID-19 pandemic, soils were stored separately outside over winter in aerated tubs until the experiment could be resumed in June 2020.

Plant and bacterial strain information

The early maturing soybean variety, ESG152 (“000” very early maturity group, Euralis, France) was chosen as only temperate adapted, short lifecycle varieties can be successfully grown in the UK. Plants were grown in greenhouse conditions with a 16 h day at 25 °C, 15 °C at night. The two soya-nodulating rhizobia strains used as inocula were *Bradyrhizobium diazoefficiens* strain (R1-9) with 99.99% average nucleotide identity (ANI) to *B.*

diazoefficiens SEMIA 5080 and *Sinorhizobium fredii* strain (495) with 98.26% ANI to *S. fredii* HH103 (and *S. fredii* CCBAU 45436). The *B. diazoefficiens* strain was isolated from soybean nodules from a field trial at The James Hutton Institute (Dundee, Scotland) inoculated with Rhizoliq, Liquifix and Euralis soybean inoculant products (Maluk *et al.*, 2023). The *S. fredii* strain was isolated from soybean nodules in a greenhouse-trapping experiment at the James Hutton Institute using alkaline (Karst limestone) soil from soybean-growing areas in China. These strains were selected as the best performing symbionts from their species based on a preliminary experiment assessing the plant benefits provided by a panel of *Bradyrhizobium* and *Sinorhizobium* symbionts in a sterile pot experiment, genomes can be found on NCBI (R1-9; SAMN39830709, 495; SAMN39830710 BioSample accessions). For inoculation, rhizobia were grown in tryptone yeast broth (Howieson and Dilworth, 2016) for 7 and 3 days for *B. diazoefficiens* and *S. fredii*, respectively, and standardised to ~10^7 CFU mL^{-1}. Cultures were pelleted and resuspended in sterile buffer solution (10 mM $MgSO_4$ and 0.01% Tween-40) for plant inoculation. Control treatments were mock inoculated with sterile buffer.

Experimental design

Soil from each field was homogenised separately, combined with twice autoclaved sand at a ratio of 4:1 and passed through a 5 mm sieve to fill 9 L pots. Each soil (soya and no-soya) was split into 4 inoculation treatments (Figure 1): a control mock-inoculated treatment (C), a control supplemented with N fertiliser (N), NH_4NO_3 equivalent to 100 kg N ha^{-1} applied at 0 and 30 d (Argaw, 2014), a *B. diazoefficiens* (B) and a *S. fredii* (S) inoculant treatment, each with 5 replicates. Initial starting microbiome samples of each bulk soil were taken on the day of planting (T0). Seeds were surface-sterilised, by shaking in 2.5% (active chlorine) NaOCl for 10 minutes, then washed with sterile dH_2O six times. Three seeds per pot were planted and inoculated (1 mL of standardised bacterial- or control inoculum). Seedlings were thinned to one plant per pot 9 d after establishment. Soil was sampled from pots at 2, 22, 63 and 84 days post treatment (dpt). The soil coring strategy reflected plant size, with the first close to the plant (1 cm), then spiralling out (by 4 cm) clockwise at each time point, as the plant root

systems grew. Soil was sampled to the full depth of the pot (20 cm), homogenised, and samples taken for DNA extraction (0.5 g stored at -80 °C) and soil physicochemical analysis. The first time point was at seedling emergence (VE), the remaining time points corresponded to the following growth stages: 22 dpt = vegetative growth stages (V2 -V3), 63 dpt = pod formation (R3- R5), 84 dpt = pod fill (R6) (Purcell, Salmeron and Ashlock, 2014). At 84 dpt, plants were harvested, nodules removed, counted and seven root nodules per plant surface-sterilised (1 min. in 70% EtOH, 3 min. 10 % bleach, 6 sterile H_2O washes) and crushed in 750 µL of sterilised 10 mM $MgSO_4$ and 0.01 % tween solution (Howieson and Dilworth, 2016). A 100 µL aliquot was combined with 100 µL of 30% glycerol and stored for DNA extractions at -80 °C, while 5 µL was also streaked onto yeast mannitol agar plates containing 0.025 % congo red (Howieson and Dilworth, 2016). These plates grew at 28°C for 5 d, after which different colony morphologies were repeatedly streaked until single isolates were obtained. Plant biomass was partitioned and dried at 80 °C for 48 h until a stable dry weight was achieved to characterise plant growth and symbiotic traits. Three pots per soil type were established for trapping rhizobial symbionts using the same cultivar. Nodules were harvested 5 weeks after sowing and rhizobia extracted using the methods above.

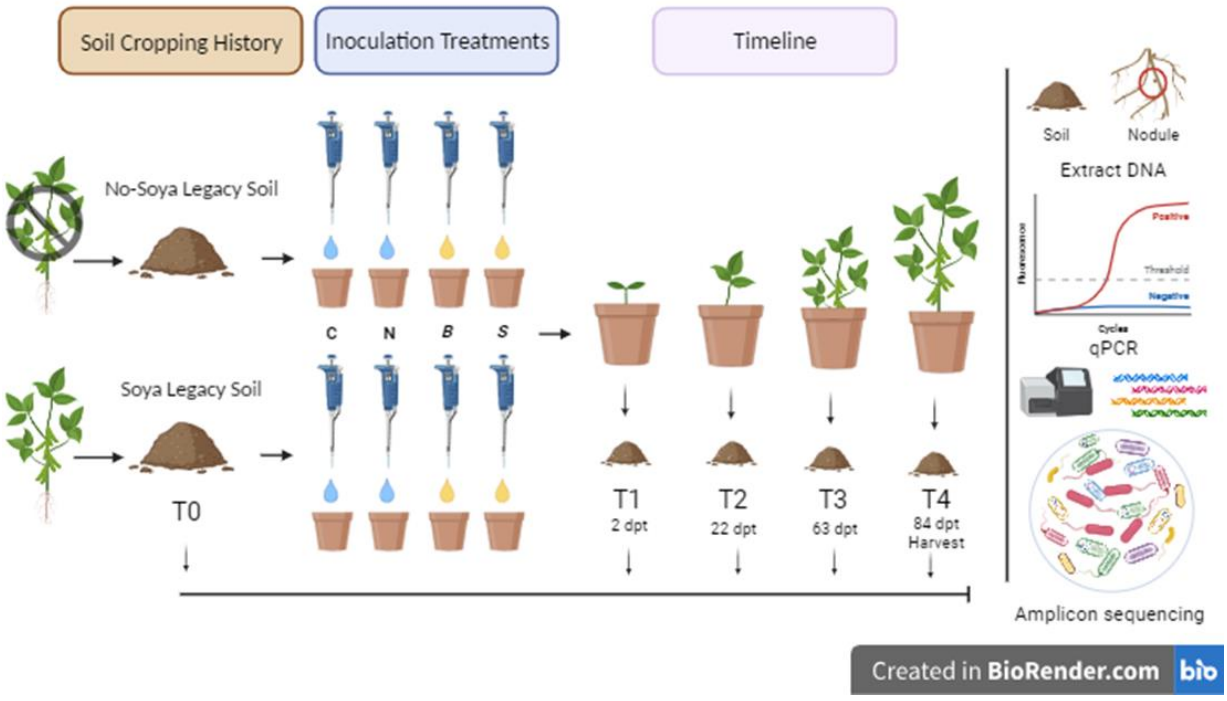


Figure 1. Experimental design and methods employed to track inoculant abundance and microbiome dynamics throughout a soybean growing season. Treatments correspond to; C = control, N = Nitrogen fertilised control, B = *Bradyrhizobium diazoefficiens* inoculant, S = *Sinorhizobium fredii* inoculant. Created with Biorender.com

Soil physicochemical analysis

At each time point, soil pH, moisture content, ammonium and nitrate levels were measured (Table S1). Soil pH and moisture content was determined by standard methods listed in (Klute, 2018). For ammonium and nitrate, 10 g of soil was combined with 40 mL 2M KCL, shaken for 1 h at 200 rpm, 25°C (230VAC Incubated Shaker, Korea), filtered through Whatman filter paper N$^{o.}$ 42 and filtrates stored at -20 °C. Soil ammonium (Baethgen and Alley, 1989) and nitrate (Miranda, Espey and Wink, 2001) concentrations were determined by colorimetric methods adjusted for a microtitreplate format (Tecan, SparkControl) and incubated at room temperature for 30 minutes (ammonium) and 2 hours (nitrate).

DNA extraction

DNA was extracted with the Machery-Nagel Nucleospin Soil kit following kit protocol (Machery-Nagel, Düren, Germany). To standardise copy number variation due to DNA extraction, 10^9 copies of a mutated *E. coli* 16S rRNA fragment per reaction was added to the starting buffer (SL2) (Daniell *et al.*, 2012). Quantity and quality of the soil DNA samples were checked on a Nanodrop 8000 (Nanodrop™), while nodule DNA samples were checked on a Qubit 4 fluorometer (Qubit™).

Primer design

Primers were designed to targe*t Alphaproteobacteria* RNA polymerase subunit B (*rpoB)* to gain insight into changes in *Alphaproteobacteria* rhizobial community dynamics (Table S2) and in particular *Bradyrhizobium* species as the 16S rRNA gene is highly conserved in this group (Joglekar *et al.*, 2020) and *rpoB* is one of the most diverse core genes (Ogier *et al.*, 2019). The *rpoB* gene was extracted from 138 soil-dwelling bacteria from the NCBI gene

database (O'Leary *et al.*, 2016), 82 residing within the *Alphaproteobacteria*, 56 spanning *Betaproteobacteria* and other major bacterial phyla such as *Actinobacteria*, *Bacteroidetes* and *Firmicutes* (Saha *et al.*, 2019) and the inoculant genomes. Genes were aligned using MUSCLE (Edgar, 2004) and a NJ phylogenetic tree with the evolutionary model F84 + G was run (100 bootstraps) on Topali (Milne *et al.*, 2004). Primers were designed by eye and using Primers4Clades (Contreras-Moreira *et al.*, 2009). Primers were optimised and to confirm primer specificity, tested on a panel of bacterial DNA extracts including the inoculant strains and other rhizobial and non-rhizobial strains (*B. diazoefficiens* USDA110^T, *B. japonicum* USDA6^T, *B. elkanii* USDA76^T, *B. ottowaense* HAMBI3284^T, *B. yuanmingense* LMG21827^T, *S. fredii* HH103, *S. meliloti*^{T} LMG6133, *S. medicae*^{T} LMG6133, *Rhizobium leguminosarum* sbv. *viciae* genospecies B, *Rhizobium leguminosarum* sv. *viciae* genospecies C, *R. etli*^{T} CFN42, *Mesorhizobium ciceri*^{T}, *Pseudomonas flurorescens* SWB25, *P. putida* F1, *Sphingobacterium sp*. and *Flavobacterium johnsoniae*). Separate primer sets for the *B. diazoefficiens* and *S. fredii* symbiotic gene *nodZ* were designed, as it has been suggested to be one of the most diverse symbiosis genes (Tian *et al.*, 2012; Ormeño-Orrillo *et al.*, 2013). The *nodZ* gene was extracted from 45 *Alphaproteobacteria* rhizobia genomes from NCBI and the inoculant genomes, aligned with the above algorithms, then primers were designed by eye. The *nodZ* gene of the *Bradyrhizobium* soybean inoculant strains, *B. japonicum* SEMIA 5079 and *B. diazoefficiens* SEMIA 5080 is 100% identical, allowing for it to be used as a proxy to track the soya-nodulation function across these species, however the designed *rpoB* primers targets a region that delineates the two. The *nodZ* primer sets were tested on the panel of rhizobial DNA mentioned above and showed specificity only to the *B. diazoefficiens/ B. japonicum* and *S. fredii* strains respectively.

Nodule isolate strain identification

Colony BOX-PCR was conducted on the nodule isolates at the end of the experiment (Table S2). The BOX primer amplifies palindromic regions of DNA, allowing discrimination between

isolates (Versalovic *et al.*, 1994). DNA products were visualised on a 2% Agarose gel in Tris-borate-EDTA (TBE) buffer stained with SYBRsafe dye, run at 90 V for 110 min.

Temporal tracking of inoculant rhizobia

Relative gene copy numbers of 16S rRNA (Muyzer, de Waal and Uitterlinden, 1993), *Alphaproteobacteria rpoB, Bradyrhizobium* soya-nodulating *nodZ* and *S. fredii nodZ* were estimated via quantitative PCR. Primers targeting the *E. coli* spike DNA was used to estimate relative copy numbers of each target (Table S2). Standards for each target were created by cloning PCR product into pGEMT-easy vector following the manufacturer's instructions (Promega, UK). Serial dilutions were made in the concentrations ranging from 10^8 to 10^2 copies μL^{-1} and run in triplicates alongside DNA samples on the Lightcycler 480 (Roche Diagnostic Systems, UK). As *nodZ* was in low abundance and PCR becomes more prone to errors with increasing cycles, samples that had a nonspecific melt curve and came up later than a set threshold (cp 45) were removed. The *Sinorhizobium nodZ* target was also run on a gel after qPCR to ensure presence of a single 186 bp product. Relative quantification compares the levels of two different target sequences within a single sample (e.g. spike vs *nodZ*) and expresses the result as a ratio. Relative gene copy numbers per gram dry weight soil were then calculated by:

1. (spike added per gram soil; 10^9) x (reference:target ratio) / dry weight of soil sample

Tracking community dynamics following inoculation

PCRs for amplicon sequencing were conducted on soil DNA (16S rRNA (Caporaso *et al.*, 2012) and *rpoB)* and nodule DNA (*rpoB*) using the primers and cycling conditions listed in Table S2. Amplicons were cleaned, indexed and sequenced at the Centre for Genomics Research (University of Liverpool), on Illumina Miseq (2x250 bp). Raw Fastq files were trimmed for the presence of Illumina adapter sequences using Cutadapt v1.2.1 (Martin, 2011) and quality checked. Paired-end reads were joined using QIIME2 (v2021.11; Bolyen *et al.*, 2019) with the DADA2 pipeline (Callahan *et al.*, 2016) to de-noise reads and create

amplicon sequencing variants (ASVs). Sequencing depth varied per sample, therefore rarefaction to 33,175 sequences for 16S rRNA and 1,567 sequences per sample for the *rpoB* dataset resulted in minimal sample losses and gave good representation (Figure S1). After initial assessment of the Greengenes, Silva and NCBI 16S RefSeq databases, the NCBI taxonomy database classified more 16S rRNA reads to the genus level for the focal genera of this study (*Bradyrhizobium* and *Sinorhizobium*). Therefore, the 16S rRNA dataset was trained on the NCBI 16S rRNA gene RefSeq dataset (O'Leary *et al.*, 2016), downloaded, and assigned taxonomy using RESCRIPt (Li *et al.*, 2021) in QIIME2 (Bolyen *et al.*, 2019).

A *rpoB* dataset was curated by downloading 20,000 *rpoB* sequences spanning bacterial taxonomic groups from the JGI IMG database (Chen *et al.*, 2021). This dataset was converted into a nucleotide NCBI database against which the *rpoB* ASVs generated in this study were Blasted, to return output format 6, max E-value 0.0001 and the top 5 Blast hits. All the top Blast hits were classified within *Alphaproteobacteria*, and hence a new dataset was created with all available *Alphaproteobacteria rpoB* sequences to increase chances of better classification (7,491 *rpoB* sequences 12/04/2022). The *Alphaproteobacteria rpoB* database was imported into QIIME2, a taxonomy classifier was trained using the *rpoB* primer extracted reads, and taxonomy was assigned to ASVs with confidence values. For tracking the inoculants in the soil microbiome, extracted *rpoB* amplicons from the inoculant genomes R1-9 (*B. diazoefficiens*), 495 (*S. fredii*), *B. diazoefficiens* SEMIA 5080 (RefSeq: NZ_ADOU00000000.2), *B. japonicum* SEMIA 5079 (Refseq: NZ_CP007569.1) were Blasted against the *rpoB* representative sequences to find their corresponding ASV in the ASV abundance table.

Data analyses

Data were analysed on R (v4.1.3) with Rstudio (R Studio Team, 2020). Biomass, symbiotic traits and qPCR data were analysed using packages in tidyverse (Wickham *et al.*, 2019). For

plant biomass and symbiotic traits, two- way ANOVAs assessing the impact of soil type and inoculation treatment were performed after checking the models conformed to the test assumptions. Nodule investment was calculated as (nodule biomass / aboveground biomass) to give a proportion of how much plants invested into the symbiosis and was arcsine square root transformed before statistical analysis. Tukey post-hoc comparison tests were employed to find groups that significantly differed. Coefficients (*t* and *p*- values) within the linear models are compared to the control (-N) unless stated otherwise and are provided to support specific treatment effects. Linear mixed effects models investigating the fixed effects soil, treatment, dpt and their three-way interactions, accounting for repeated measures (1| pot.id), were constructed for tracking the symbiosis gene *nodZ* (log transformed) and alpha diversity over time in the soil microbiome (estimated using REML and nloptwrap optimizer). Linear mixed effects models were made with package lme4 (Bates *et al.*, 2015) and assessed using the 'anova' function (base R) for model comparisons and the 'Anova' function in the car package (Fox and Weisberg, 2019) for assessing the effect of independent variables and *p*-value generation.

16S rRNA and *rpoB* amplicon datasets were assessed using the phyloseq (McMurdie and Holmes, 2013) and vegan (Oksanen *et al.*, 2020) R packages. As diversity levels differed between soils, one-way ANOVAs within soil types were performed to assess the effect of inoculation treatment within time points and Tukey post-hoc comparisons used to determine significantly different groups. Weighted Unifrac and Bray-Curtis distance matrices were assessed for beta- diversity measures, with only Bray- Curtis presented as results were similar. A permutation test for the homogeneity of multivariate dispersion was run prior to testing for the impacts of soil, treatment and their interaction on distance matrices by PERMANOVA with 999 permutations. Where the assumption of multivariate dispersion homogeneity was not met, the non-parametric statistical test ANOSIM was used. A constrained analysis of principal coordinates (CAP) was conducted to assess the impacts of

soil traits, pH, soil moisture content, NH^{4+} and NO_{3-} and dpt on distance matrices of bacterial communities.

2.4 Results

Soil history and inoculation influences plant growth and symbiotic traits

Plant biomass and symbiotic traits varied depending on the soil history and inoculation treatment (Figure 2). Surprisingly, the control in the soya history soil yielded the largest total (Figure 2A; ANOVA, treatment:soil, $F_{3,32} = 5.8$, $p < 0.01$) and seed biomass (Figure 1B; ANOVA, treatment:soil, $F_{3,32} = 6.3$, $p < 0.01$), which was significantly larger than the no-soya soil control and the *Sinorhizobium* treatment in the soya soil (Tukey test, $p < 0.05$). The *Sinorhizobium* treatment in the no-soya soil and the *Bradyrhizobium* treatment in the soya soil yielded similar seed biomass to the soya soil control (Tukey test $p < 0.05$).

Exotic rhizobia induced different nodulation phenotypes depending on the soil to which they were introduced (Figure 2C and Figure 2D). All plants were nodulated in this study, which was unexpected in the no-soya control treatments, as no SNR were expected to be present in this soil microbiome. In the no-soya soil, *Sinorhizobium* inoculation induced significantly higher nodule investment compared to all other treatments (ANOVA: treatment, $F_{3, 29} = 61.5$, $p < 0.0001$), but in the soya soil this was reduced to similar levels as all other treatments (ANOVA: treatment:soil, $F_{3, 29} = 37.1$, 13, $p < 0.0001$, Tukey test $p < 0.05$). *Bradyrhizobium* inoculation in the no-soya soil increased nodule investment compared to the nitrogen control ($t = 3.7$, $p < 0.001$), however did not significantly change nodule investment in the soya soil. There is a clear inoculation legacy effect in the soya-soil, as nodule numbers were significantly higher in all treatments than the no-soya soil (ANOVA: soil $F_{1, 32}= 14$, $p < 0.001$), except for the *Sinorhizobium* treatment (ANOVA: treatment $F_{3, 32} = 37$, $p < 0.001$), which drove a significant treatment: soil interaction (ANOVA: $F_{3, 32} = 18.2$, $p < 0.001$). Average percentage increase of nodules in the soya soil treatments compared to the no-

soya soil control, nitrogen and *Bradyrhizobium* treatment were 575%, 1078% and 550%, respectively.

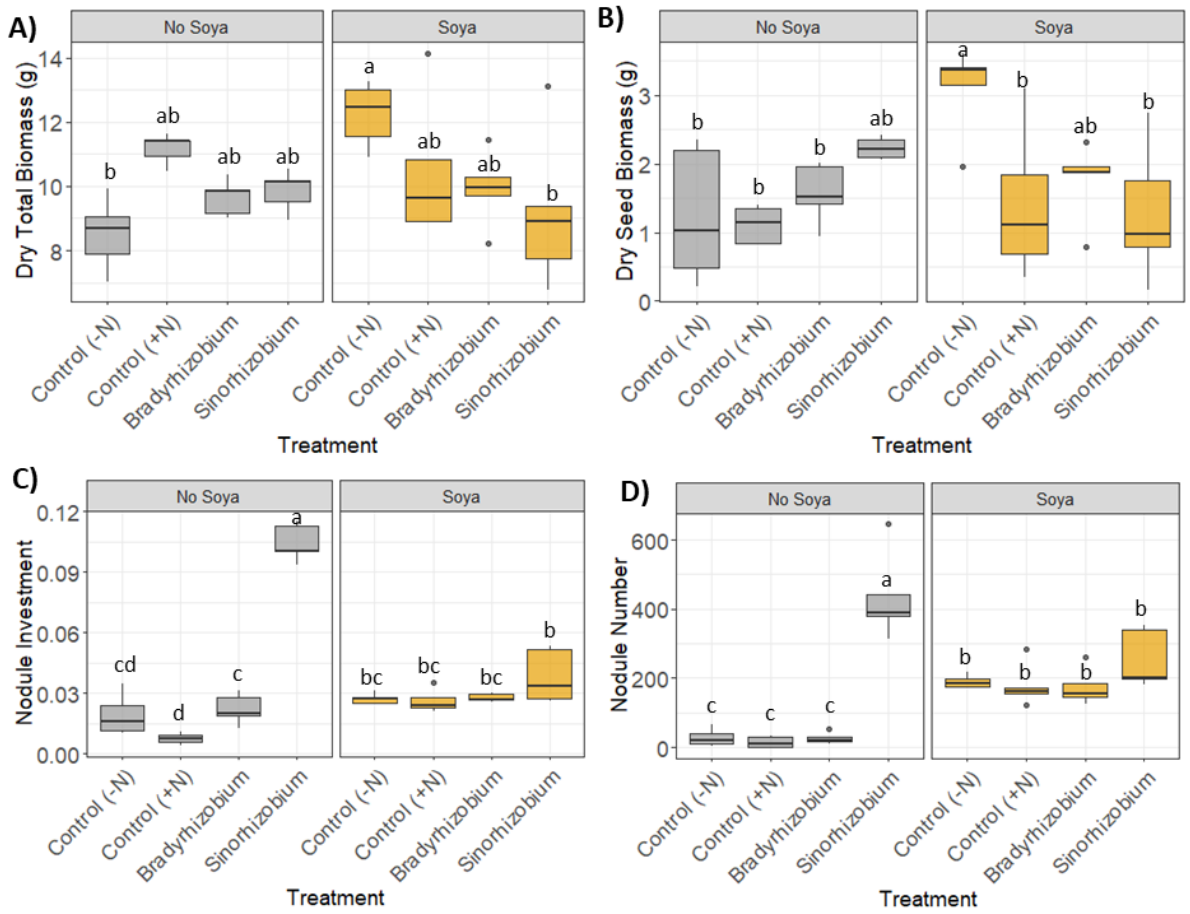


Figure 2. Plant biomass and symbiotic traits are affected by a combination of soil inoculation history and exotic rhizobial inoculation. **(A)** total biomass, **(B)** seed biomass, **(C)** nodule investment and **(D)** nodule numbers under different inoculation treatments in the soils with no- soya inoculation history or soya inoculation history (n = 5). Letters denote statistical significance from Tukey HSD tests where $p < 0.05$.

Tracking inoculant abundance in the soil microbiome revealed the widespread prevalence of previous Bradyrhizobium japonicum inoculant

Soya-nodulating symbionts were tracked in the microbiome by qPCR of the symbiosis gene *nodZ* and amplicon sequencing of the core gene *rpoB* (Figure 3). *Bradyrhizobium nodZ* was detected in both bulk soil microbiomes at the start of the experiment (T0). Relative gene copy counts of *Bradyrhizobium nodZ* in the no-soya soil indicated a starting population of

1.42 x 10^4, compared to 2.25 x 10^5 in the soya soil, which was 15.7-fold higher (Welch two sample T-test, $t_{4.26}$ = -5.6, p = < 0.01). This suggests that the presence of nodules found in the no-soya control treatments came from pre-existing populations. Evidence from the *rpoB* amplicon sequencing data suggests this is due to the presence of *B. japonicum* (Figure 3), which formed part of the previous inoculant introduced to the soya soil on farm, which appears to have spread onsite to the uninoculated no-soya soil. *B. japonicum* increased over time in both soil microbiomes but reached a larger population in the soya soil and dominated nodule occupancy in all treatments, bar the *Sinorhizobium* treatment in the no- soya soil, despite not being directly inoculated in this trial (Figure 3). Furthermore, 32 out of 34 nodule isolates obtained at harvest were *B. japonicum* SEMIA 5079, with only 2 isolates identified as *B. diazoefficiens* SEMIA 5080 (Figure S2). Isolates from trap plants grown in parallel to the experiment also highlighted the sole presence of the *B. japonicum* strain SEMIA 5079 in the no-soya soil, whereas both symbionts (*B. japonicum* SEMIA 5079 and *B. diazoefficiens* SEMIA 5080) were isolated from trap plants in the soya soil (Figure S2).

Fluctuations in *nodZ* mirror patterns in inoculant *rpoB* relative abundance in the soil microbiome (Figure 3). *Bradyrhizobium nodZ* remained higher in the soya soil throughout the experiment (ANOVA, type II: soil χ^2 = 59.1, *d.f.* = 1, p < 0.0001), but was elevated at 2 dpt after *B. diazoefficiens* inoculation in both soil microbiomes and at 63 dpt during a peak in *B. japonicum* abundance in the soya soil control treatment (ANOVA, type II: treatment:dpt χ^2 = 25.95, *d.f.* = 3, p < 0.0001). No *Sinorhizobium nodZ* was detected in either soil prior to inoculation, and by the end of the experiment was only observed in the *Sinorhizobium*-inoculated soil microbiomes. Similarly, inoculation induced a large increase in *S. fredii nodZ* at 2 dpt, which then decreased by 22 dpt, but remained at stable levels in the soil throughout the experiment (ANOVA, type II: treatment:dpt χ^2 = 15.31, *d.f.* = 3, p < 0.01), which was reflected by *S. fredii rpoB* counts. When inoculated into the no-soya soil *S. fredii* was the most abundant symbiont in the nodules (76% relative abundance) but was far less abundant in the soya-soil root nodules (3.9% relative abundance), despite it being at a similar

population size in both soil microbiomes. In the soya-soil, *B. diazoefficiens* was found in the nodules of all treatments (as it formed part of the previous inoculant) and (re)inoculation in the soya soil *Bradyrhizobium* treatment, didn't increase its abundance in nodules. Reads that were assigned to *B. elkanii rpoB* were found within one replicate of the nitrogen control treatment nodules in the no-soya soil (1.6% relative abundance). To our knowledge, *B. elkanii* was not a component of the previous inoculant applied on farm (Legume Technology Ltd), nor in this study.

Apart from immediately after inoculation (2 dpt), the inoculant species (*B. diazoefficiens* and *S. fredii*) were rare in the soil microbiomes. Total bacterial (16S rRNA) and *a*-proteobacteria (*rpoB*) populations quantified by qPCR, revealed similar population sizes between the two soils and were unaffected by exotic inoculation treatments (data not shown). Taken together, this highlights how previous soya cropping and inoculation has established a SNR population in the soya-soil field, from which the symbiont *B. japonicum* has spread and persisted in the no-soya soil field and dominated nodule communities when soybean was grown and inoculated with different strains. Exotic rhizobia fluctuated in the soil microbiomes and remained in the soil by the end of the experiment.

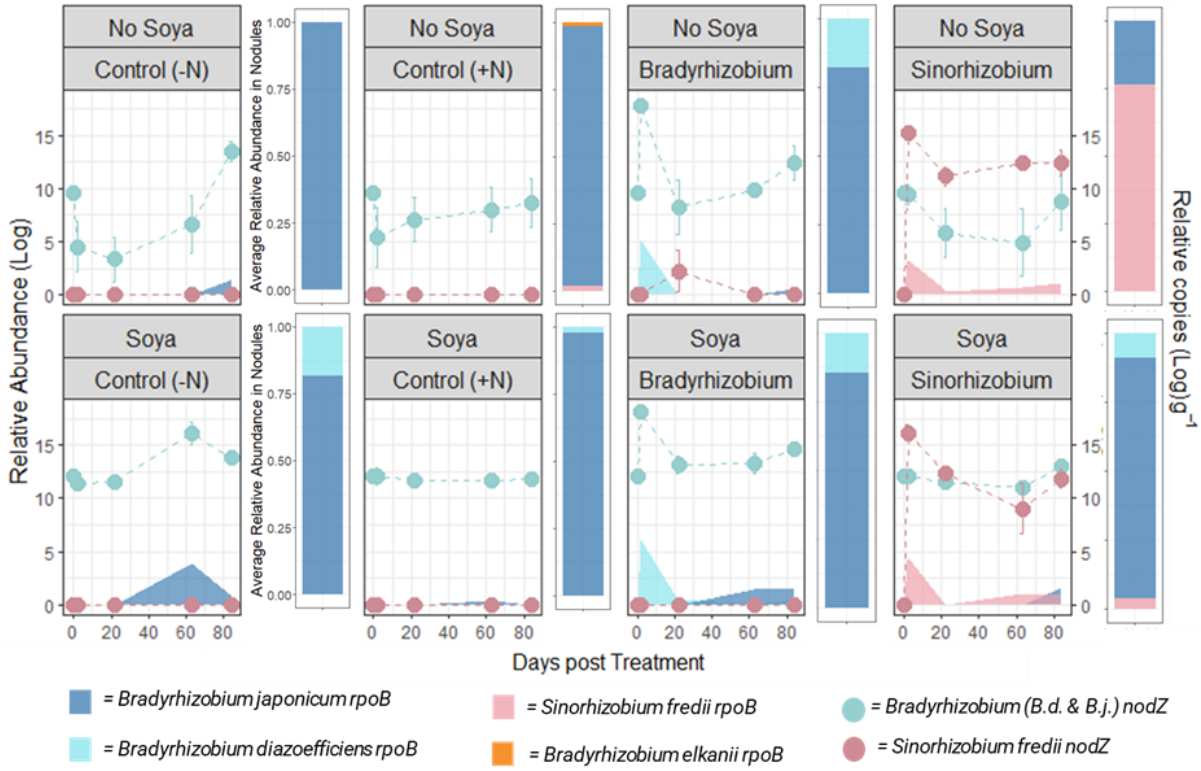


Figure 3. Widespread presence of previous inoculant *B. japonicum* in the soil microbiome and in nodules at harvest. Fluctuations in *nodZ* mirror patterns in inoculants *rpoB*. Coloured areas relate to average log *rpoB* counts from rarefied ASV table (left y-axis, dark blue = *B. japonicum*, light blue = *B. diazoefficiens*, pink = *S. fredii*, *n* = 5). Blue circles are average relative copies of *Bradyrhizobium* (*B. japonicum* and *B. diazoefficiens*) *nodZ* g^{-1} dry weight soil, error bars are +/- standard error, pink circles are average relative copies of *S. fredii nodZ* g^{-1} dry weight soil, +/- standard error, (right y-axis, *n* = 5). Stacked bar charts to the right of treatment panels are the respective proportion of symbionts found in root nodules at harvest, averaged across 5 replicates, with the same colour scheme as above, plus orange = *Bradyrhizobium elkanii rpoB*. Top panels = no- soya soil microbiome, bottom panels = soya soil microbiome.

Inoculation induces transient impacts on soil bacterial communities

Overall, soil bacterial diversity and composition was mostly influenced by soil type and temporal effects (dpt), but exotic rhizobial inoculation did alter soil communities immediately after inoculation, in a species-specific manner (2 dpt), and during peak nitrogen fixation (63 dpt). Soil inoculation history had an impact on *Bradyrhizobium* and *Alphaproteobacteria*

Shannon's diversity, which was significantly higher in the soya bulk soil for both the *Bradyrhizobium* community (Wilcoxon Rank Sum test, w = 0, $p < 0.01$) and *Alphaproteobacteria* community (Wilcoxon Rank Sum test, w = 0, $p < 0.001$), and remained higher over the course of the experiment (Figure 4A, *Bradyrhizobium* community, ANOVA type II: soil, $\chi^2 = 26.19$, *d.f.* = 1, $p < 0.001$, *Alphaproteobacteria* community, ANOVA type II: soil $\chi^2 = 34.88$, *d.f.* = 1, $p < 0.001$). Whereas for the whole bacterial community, the two soils had similar starting Shannon's diversity, but over the course of the experiment, diversity declined in all treatments in the no-soya soil but not in the soya- soil (Figure 4A; ANOVA, type II: soil:dpt interaction, $\chi^2 = 5.03$, *d.f.* = 1, $p < 0.05$).

As diversity levels differed between soils, the impact of inoculation treatment was assessed within soil types at timepoints of interest. In the soya soil, inoculation with *B. diazoefficiens* caused a large decline in diversity at 2 dpt in the *Bradyrhizobium* community (ANOVA; treatment, $F_3 = 18$, $p < 0.001$; t = -6.4, $p < 0.001$) which was also observed at the *Alphaproteobacteria* class level (ANOVA; treatment, $F_3 = 10.9$, $p < 0.001$; $t = -5.7$, $p < 0.001$) highlighting how inoculant interactions within *Bradyrhizobium* were driving diversity fluctuations seen at the class level. (Figure 4A). A smaller decline in diversity was also seen in the no-soya soil at 2 dpt in the *Bradyrhizobium* community (ANOVA; treatment, $F_3 = 3.3$, $p < 0.05$; $t = -3$, $p < 0.01$), which was not significant at the *Alphaproteobacteria* level (ANOVA; treatment, $F_3 = 2.7$, $p = 0.08$), but coefficients within the linear model highlighted that the *Bradyrhizobium* treatment was significantly less diverse than the control treatment in this soil ($t = -2.53$, $p < 0.05$). The drop in diversity in the *Bradyrhizobium* treatment at 2 dpt was not seen at the whole bacterial community level (Figure 4A). At 2 dpt, both *B. diazoefficiens* and *S. fredii* were significantly enriched within their inoculated treatments, but the effect (F-statistic and *p*- value) of *B. diazoefficiens* was larger (Figure S3). Significant enrichment of the inoculant species at 2 dpt corresponds with the spikes in abundance observed in Figure 3, the relative abundance of *B. diazoefficiens* in Figure 4C and *Bradyrhizobium* and *Sinorhizobium* genera in Figure 4D. Despite *S. fredii's* abundance at this time point, the

Sinorhizobium treatment did not induce a decline in diversity in the *Alphaproteobacteria* communities like the *Bradyrhizobium* treatment. *Bradyrhizobium* was the most abundant genus within *Alphaproteobacteria* (Figure 4D), whereas *Sinorhizobium* were rare, with *S. fredii* the only species detected.

B. diazoefficiens inoculation shifted *Bradyrhizobium* and alphaproteobacterial community composition in both soil microbiomes at 2 dpt (Figure 4B). Communities from the same soil were significantly more similar (*Alphaproteobacteria* community ANOSIM; R = 0.95, $p < 0.001$, *Bradyrhizobium* community ANOSIM; $R = 0.68$, $p < 0.001$) but inoculation treatment also significantly impacted the Bray- Curtis dissimilarity matrix (*Alphaproteobacteria* community, ANOSIM, $R = 0.14$, $p < 0.05$, *Bradyrhizobium* community ANOSIM; $R = 0.26$, $p < 0.001$). No such change was observed for *S. fredii* treatments. Differences in community composition at 2 dpt were not evident in later time points, where only a significant influence of soil type on beta-diversity remained (data not shown). The rarefaction level (1,597 sequences per sample) removed 3 out of 5 replicates within the soya-soil, *Bradyrhizobium,* 2 dpt time point. As this treatment and time point highlighted interesting changes in the microbiome, analyses were repeated to a lower sequencing depth to retain a third replicate (Figure S4). All reported findings were upheld and inoculation treatment had a significant impact on *Alphaproteobacteria* Shannon's diversity at 2 dpt in the no-soya soil (Figure S4, ANOVA; $F_3 = 5.3$, $p < 0.01$), where *B. diazoefficiens* inoculation significantly reduced Shannon's diversity in the no- soya soil microbiome ($t = -3.1$, $p < 0.01$) as well as in the soya soil microbiome (Figure S4, ANOVA; $F_3 = 7.4$, $p < 0.01$, $t = -4.1$, $p < 0.01$).

The time point 63 dpt corresponded with the early pod-fill plant growth stage (R3-R5), when soybeans were undergoing peak N_2 fixation (Ciampitti *et al.*, 2021). At 63 dpt, there was a significant effect of inoculation treatment on whole bacterial Shannon's diversity in the no- soya microbiome (ANOVA, $F_3 = 5$, $p < 0.05$), where the *Bradyrhizobium* treatment was significantly lower compared to the controls (control: $t = -2.9$, $p < 0.05$, nitrogen: $t = -3.3$, $p <$

0.01) and the *Sinorhizobium* treatment significantly reduced diversity compared to the nitrogen control (t = -2.4, p < 0.01; Figure 4A). Differences between treatments were not evident in the soya-soil microbiome (Figure 4A). Soil type explained the largest amount of variation for Bray- Curtis distances (Figure 4B, PERMANOVA; soil, F_1 = 18.1, R^2 = 0.31, p < 0.001), but inoculation treatment further modified microbial community composition (PERMANOVA; treatment, F_3 = 1.6, R^2 = 0.08, p < 0.05). When grouped by inoculated (*Bradyrhizobium* and *Sinorhizobium*) or not (N- and N+ controls), there was a significant effect of inoculation on Bray- Curtis distances (PERMANOVA; F_1 = 2.68, R^2 = 0.04, p < 0.05) suggesting that rhizobia inoculation, regardless of the species, shifted community composition at 63 dpt, resulting in more similar communities compared to the mock-inoculated controls (Figure 4B). Significantly changed genera at 63 dpt highlighted that N fertilisation resulted in the most abundance changes at the genus level (Figure S3) and *Bradyrhizobium* was only significantly enriched in the soya-soil control treatment (corresponding with the increase in *B. japonicum* at 63 dpt; Figure 3). This suggests that changes in community composition in inoculated treatments were not due to an increase in inoculum abundance but were the result of changes in many bacterial groups (Figure S3). *Alphaproteobacteria* comprise a relatively small component of the bacterial communities and there are only subtle changes visible in other bacterial classes (Figure 4D). The differences seen at this time point disappeared by the end of the experiment (84 dpt), where only a significant impact of soil type on Bray- Curtis distances remained (data not shown). To investigate what soil factors may be contributing to the differences between bacterial communities a constrained analysis of principal coordinates assessing effects of soil pH, NH^{4+}, NO_{3-}, soil moisture content (SMC) and dpt on the bacterial communities over the experiment was conducted. Soil pH, SMC and dpt all had significant impacts on bacterial (16S rRNA) and rhizobial (*rpoB*) community composition, whereas NH^{4+} and NO_{3-} levels had no overall effect (Figure S5). Soil pH was notably higher in the no-soya soil (7.2) than the soya soil (6.8; Table S1) and remained higher throughout the experiment (data not shown).

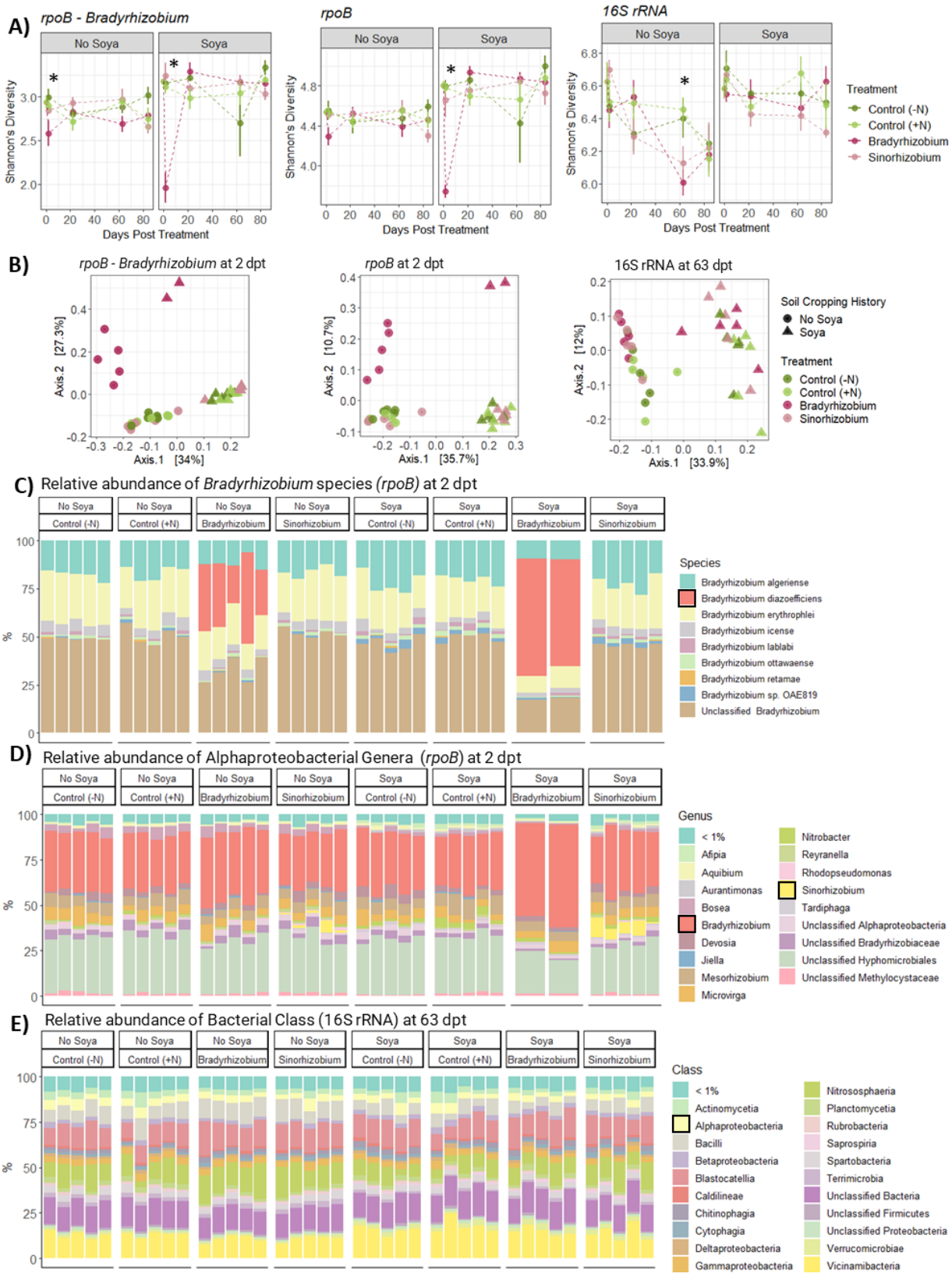


Figure 4. Inoculation alters soil bacterial community diversity and composition transiently. **A)** Shannon's diversity during the experiment for the genus *Bradyrhizobium* (*rpoB* - left), class

Alphaproteobacteria (*rpoB* - centre) and Bacterial community (16S rRNA - right). Coloured points are averages with standard error bars, colours correspond to inoculation treatments: dark green = Control (-N), light green = Control (+N), dark pink = *Bradyrhizobium*, light pink = *Sinorhizobium.* Black stars denote time points where significant differences between treatments were found. **B)** Principal Coordinates Analysis of Bray- Curtis Dissimilarity Matrix for genus *Bradyrhizobium* at 2 dpt (*rpoB* - left), class *Alphaproteobacteria* at 2 dpt (*rpoB* - centre) and bacterial community at 63 dpt (16S rRNA - right). Colours correspond to the inoculation treatments as listed above, circle = no-soya soil, triangle = soya soil. **C)** Relative abundance of *Bradyrhizobium* (*rpoB*) species at 2 dpt, black square highlights inoculant species. **D)** Relative abundance of *Alphaproteobacteria* genera (*rpoB*) at 2 dpt, black square highlights inoculant genera. **E)** Relative abundance of Bacteria (16S rRNA) at 63 dpt, black square highlights inoculants class.

2.5 Discussion

This study investigated the impact of inoculating two exotic rhizobia species, on two soil microbiomes with or without a soybean cultivation history from a UK farm. A legacy effect of previous soya growth and inoculation was found, which, when left uninoculated, resulted in the best plant biomass traits. The inoculant strains previously introduced on site had persisted in the soil microbiomes, with widespread prevalence of *B. japonicum* SEMIA 5079 within nodule communities. Soil bacterial communities were altered transiently under inoculation treatments, with *B. diazoefficiens* affecting the *Bradyrhizobium* and *Alphaproteobacteria* community two days post inoculation, and both inoculant species altering bacterial community dynamics during early pod fill (63 dpt). Soil type and temporal effects throughout plant growth had a larger effect on bacterial communities than inoculation, potentially suggesting that inoculation impacts may be transient in the soil microbiome, even though the introduced function (i.e. soya-BNF) persists.

Inoculation legacy on plant biomass and symbiont populations

Overall, there was no consistent impact of rhizobia inoculation on plant biomass in this trial, however the best plant biomass yields were found in the soil where soya was previously grown and inoculated, which was reduced when N fertiliser or the exotic *S. fredii* strain was inoculated. This suggests a high BNF potential due to the carry-over of inoculants in this soil.

Inoculation often improves crop productivity when there is a low or absent background population of compatible indigenous rhizobia present in the soil microbiome (Denton *et al.*, 2002, 2003; Thilakarathna and Raizada, 2017), as is currently the case for soybean in the UK, but in this study, the impact of inoculation may have been confounded by the presence of inoculant rhizobia (*B. japonicum*) in the non- inoculated soil. The prevalence of *B. japonicum* SEMIA 5079 across field soils suggests that it has spread to areas which have not received inoculated seed. Soil for this experiment was sampled after the third consecutive year that soybean cv. Siverka (SoyaUK) and its *Bradyrhizobium* inoculants (Legume Technology Ltd.) had been sown on this farm in three different fields. Therefore, spread via agricultural machinery, ground water, and/or wind, plus the high saprophytic capability of the inoculant strain (Siqueira *et al.*, 2014), may explain the presence in the no-soya soil microbiome. *B. japonicum* SEMIA 5079 is a natural variant of *B. japonicum* SEMIA 566, belonging to the highly competitive serogroup USDA 123 (Siqueira *et al.*, 2014). The competitiveness and persistence of these strains has been evidenced in Brazilian soils, where they originate (Vargas *et al.*, 1994; Hungria and Vargas, 2000; Mendes, Hungria and Vargas, 2004; Hungria *et al.*, 2006) and in European soils where they've been introduced (Damirgi, Frederick and Anderson, 1967; Moawad, Ellis and Schmidt, 1984; Obaton *et al.*, 2002; Narozna *et al.*, 2015). For example, Vargas *et al.*, (1994) found *B. japonicum* SEMIA 566 at 5 out of 6 experimental sites, dominating nodule occupancy at 3 of these sites, despite not being inoculated in these areas (Vargas *et al.*, 1994). The authors attributed the prevalence of this symbiont to potential introduction via seeds and farm machinery from southern Brazil where it was widely used in inoculants until 1978. Similarly Mendes *et al.,*

2004, highlighted that despite repeated inoculations with other strains (including *B. diazoefficiens* SEMIA 5080), strains related to the USDA 123 serocluster dominated nodule occupancy, occurring in >50% of nodules in treatments where they had never been inoculated (Mendes, Hungria and Vargas, 2004). Strains from the USDA 123 serogroup have been found to dominate in other areas where they have been introduced, regardless of soil type or host genotype (Damirgi, Frederick and Anderson, 1967; Moawad, Ellis and Schmidt, 1984; Obaton *et al.*, 2002) and have been found to persist in the soil for up to 17 years without further soybean cultivation after the original introduction (Narozna *et al.*, 2015). The *B. diazoefficiens* strain used as the inoculant in this study (a natural variant of *B. diazoefficiens* SEMIA 5080) is known for its elite nitrogen fixation ability (Siqueira *et al.*, 2014), however can be outcompeted for nodule occupancy by it's usually-introduced counterpart *B. japonicum* SEMIA 5079 (Mendes, Hungria and Vargas, 2004; Hungria *et al.*, 2006), as observed in this study. Genome analyses of these inoculant strains suggests that *B. japonicum* SEMIA 5079 contains more genes involved in secondary metabolism, nutrient transporters, iron-acquisition and auxin metabolism compared to *B. diazoefficiens* SEMIA 5080 (Siqueira *et al.*, 2014), which may contribute to its survival in soils. Inoculation into areas with naturalised soybean symbionts often results in an absence of yield benefits (Ambrosini *et al.*, 2019; Zilli *et al.*, 2021), with the exception of field trials in Brazil (Hungria *et al.*, 2006), however it is perhaps surprising that inoculation after just one season in the soya-soil did not produce a yield response and highlights how the benefits of inoculating superior N_2 fixers can be dampened if competitive symbionts are present in the soil microbiome (Mendes, Hungria and Vargas, 2004; Mendoza-Suárez *et al.*, 2021). Hypotheses from Bell and Tylianakis (2016) suggest that intensified agriculture selects for certain soil bacterial taxa, and by extension genes, which can spill over into adjacent unmodified areas (Bell and Tylianakis, 2016). This, combined with the widespread inoculation of bacteria, where populations are boosted exponentially with legume productivity and the functional symbiosis genes of interest are mobile, may increase the likelihood of microbial spill-over from

agricultural areas. These results suggest careful consideration of strain genotypes may be needed going forwards for re-inoculation strategies.

Evidence from this trial suggests that the novel inoculant *S. fredii* could outcompete SEMIA 5079 for nodule occupancy in certain soil environments. In the no-soya soil microbiome, which had a higher pH and significantly less *Bradyrhizobium nodZ*, *S. fredii* was the dominant symbiont in the nodules. However, *S. fredii* did incur a higher investment into the symbiosis. *S. fredii* inoculated plants trended towards better plant benefits in the no-soya soil, providing a 16% increase in total biomass and 100% increase in seed biomass compared to the N- and N+ controls, respectively. Although, *Sinorhizobium* species are dominant soybean symbionts in native alkaline soils (Zhang *et al.*, 2017; Han *et al.*, 2020), due to their genetic adaptations to alkaline soil conditions (Tian *et al.*, 2012), currently no commercial soybean inoculants used in the UK contain *Sinorhizobium* species. This is because *Bradyrhizobium* symbionts often outperform *Sinorhizobium* species in most acidic - neutral pH soils in terms of plant benefits (Ravuri and Hume, 1992) and outcompete them for nodule occupancy if *S. fredii* is inoculated into soils already possessing soya-nodulating *Bradyrhizobium* (Albareda, Rodríguez-Navarro and Temprano, 2009a, 2009b) as seen in the soya soil in this study. The potential benefits of this symbiont may be enhanced in agricultural soils with higher pHs (pH ≥8), offering the opportunity for tailored inoculants.

In Europe, where soybean has been grown and inoculated more widely, there is research investigating the use of naturalised strains for the inoculation of temperate soybean varieties (Yuan *et al.*, 2020; Halwani *et al.*, 2021; Van Dingenen *et al.*, 2022), where significant strain x cultivar x environment interactions have been highlighted as considerations when introducing them as inoculants (Omari *et al.*, 2022). Interestingly, a *B. elkanii* symbiont was detected in the nodules of a single plant replicate in the nitrogen control in the no- soya soil; as there was no prior knowledge that a soya- nodulating *B. elkanii* formed part of the introduced inoculants, its presence is particularly interesting. It could

either be the result of a native *B. elkanii* strain that lacks the capability to nodulate soybean and has opportunistically colonised the nodules alongside the compatible inoculant strains (Zgadzaj *et al.*, 2015; Gano-Cohen *et al.*, 2019), or has potentially acquired the soya-nodulating symbiosis genes from the introduced inoculant strains (Barcellos *et al.*, 2007; Batista *et al.*, 2007; Nandasena *et al.*, 2007). Growing soybean without inoculation in areas that have previously been inoculated in UK soils could identify symbionts in nodules that have become locally adapted to the soil conditions, which could be utilised in inoculation trials going forwards.

Inoculation induces transient effects on soil microbiomes

The *B. diazoefficiens* inoculant induced significant changes on the *Bradyrhizobium* and *Alphaproteobacteria* communities during seedling emergence (2 dpt), which was associated with an enrichment of the inoculum species in the microbiomes. A larger decrease in diversity at 2 dpt was found in the soya soil, this may be because there was an existing population of *B. diazoefficiens* in this soil and inoculation with a large dose of *B. diazoefficiens* boosted this population, potentially inflating the impact on diversity. Whereas in the no-soya soil, this symbiont was absent and so inoculation resulted in a smaller impact on diversity. Additionally, the *Bradyrhizobium* inoculant may have been better adapted to the soya soil and thus been able to survive and reproduce to higher population densities. Soybean associated microbial communities have been shown to change according to plant growth stages, with particular selection for rhizobial symbionts (Xu *et al.*, 2009; Sugiyama *et al.*, 2014, 2015; Moroenyane, Tremblay and Yergeau, 2021), for example a spike in *Bradyrhizobium* during seedling emergence has previously been observed in root endospheres (Moroenyane, Tremblay and Yergeau, 2021). In contrast, *Sinorhizobium* inoculation did not impact the *Alphaproteobacteria* community at 2 dpt, despite being significantly enriched, nor at any of the other time points. Timing of sampling may be influential here, *S. fredii* has a generation time of about 3 - 4 hrs (Weidner *et al.*, 2012), in

comparison to 6 - 12 hours for *B. diazoefficiens* (Viteri and Schmidt, 1987) therefore, *S. fredii* may have induced similar changes in the microbiome but at an earlier time point. Alternatively, the genus *Sinorhizobium* was rare in both soil microbiomes, with *S. fredii* the only species detected; whereas *Bradyrhizobium* were a large component of the *Alphaproteobacteria* community (refer to Figure 4D) and in recent years *Bradyrhizobium* have been found to be an extremely diverse and dominant genus within microbial communities (VanInsberghe *et al.*, 2015; Hollowell *et al.*, 2016; Avontuur *et al.*, 2019; Ormeño-Orrillo and Martínez-Romero, 2019). Whether the presence and abundance of more related recipient communities contributes to the overall impact an inoculant species has on a microbiome is unclear and warrants further investigation.

A shift in bacterial communities was observed under inoculated treatments during peak BNF. *Bradyrhizobium* inoculants have been found to alter bacterial and fungal communities in soybean rhizospheres in areas of native cultivation during this growth stage (Zhong *et al.*, 2019; Xu *et al.*, 2020). Studies have also shown the importance of legume host genotype and soil factors on shaping rhizobial communities (Vuong, Thrall and Barrett, 2017; Zhang *et al.*, 2017; Brown *et al.*, 2020; Han *et al.*, 2020; Lagunas *et al.*, 2023). Host plants are subject to their surrounding soil microbial communities when assembling root associated microbiota, and plant root exudation profiles stimulate colonisation of microorganisms occupying specialised niches in the rhizosphere (Mendes *et al.*, 2014; Xiao *et al.*, 2017; Sugiyama, 2019). Some of the enriched bacterial genera observed at 63 dpt have previously been associated with soybean growth (*Bradyrhizobium, Nocardioides, Nitrososphaera, Chryseomicrobium, Pseudomonas, Nibribacter, Chitinophaga* and *Stenotrophomonas* - Figure S3) (Sugiyama *et al.*, 2014; Zhong *et al.*, 2019; Bender *et al.*, 2022), suggesting that certain native soil bacteria are preferentially selected by soybean in this novel environment. Additionally, the N_2 fixation efficiency of rhizobia inoculants has recently been found to further modify host associated communities, where highly efficient N_2 fixers can alter the composition of root endosphere communities, resulting in increased micronutrient element

acquisition (Lagunas *et al.*, 2023). Differences observed at this time point suggests that inoculation can alter microbial communities, but the specific interactions within microbiomes may differ, due to the indigenous microbial community composition. For example, the rhizosphere microbial communities of soybeans grown in agricultural soil versus forest soil significantly differed in their composition (Liu *et al.*, 2019). Selection of certain microbes from indigenous available bulk soil populations also results in reduced diversity in the rhizosphere (Liu *et al.*, 2019; Zhong *et al.*, 2019). Interestingly, a decrease in total bacterial diversity over the time course of the experiment was observed in the no- soya soil, which may be associated with the growth of a novel legume in this soil, whereas previous soya growth and inoculation may have primed the soya soil for soya-associated microbial communities. Notably, differences between treatments were not evident at harvest, suggesting that microbiomes had recovered from the influx of inoculant strains, however inoculants were still present in the microbiome (Figure 3), suggesting that the introduced functional trait soya-BNF persists.

2.6 Conclusions

This study highlighted the ecological complexities associated with the introduction of exotic inoculants to the soil microbiome. The introduced inoculant strains caused transient shifts in microbial community composition and diversity within the experimental timeline, however long-term effects are unknown. Negligible effects of inoculation on plant biomass were observed, likely due to the presence of a highly competitive symbiont in the microbiomes. Nitrogen fertilisation resulted in low seed biomass, interestingly biologically fixed N has been found to better translocate to seeds, resulting in higher seed protein content, than N from inorganic fertilisers, which instead often results in higher vegetative biomass (Hungria and Neves, 1987; Ravuri and Hume, 1992; Hungria *et al.*, 2020; Garcia, Nogueira and Hungria, 2021). This highlights the benefits of optimising BNF for the sustainable production of soybean in the UK. The inoculation legacy soil provided the best yield benefits for soybean,

but considerations for re-inoculation strategies going forwards are needed to avoid a decrease in yields. Therefore, despite transient impacts on community dynamics, the introduced bacteria and functional trait (soya- symbiosis) remains in the microbiome. Future work should focus on the long term impacts of inoculant introduction e.g. via monitoring schemes (Jack *et al.*, 2021), with special focus on areas where introduction has already occurred within the UK, as these may provide the best yield benefits. Repeated growth of soya without inoculation in these regions may select for locally adapted symbionts that have diversified from the original inoculant strains, which when used as inoculants, may impact resident communities less.

2.7 Acknowledgements

I would like to thank PlantworksUK for help with sampling the soil for the experiment. I would also like to thank Legume Technology for confirming what bacterial strains were used in inoculant products and providing SEMIA strains. Amplicon sequencing data generation was carried out by the Centre for Genomic Research, which is based at the University of Liverpool.

2.8 Supplementary Information

Table S1. Soil cropping history and physicochemical properties measured at the start of the experiment. T-tests were conducted to compare soil traits between fields. Test statistics provided in the table, NS = Not significant.

Field	Cropping History	Soil type and texture	pH	Soil moisture content (%)	Nitrate (mg/Kg)	Ammonium (mg/Kg)
1 - Not exposed to soya/ SNR	2019: Spring Barley 2018: Cleared field 2017: Apple Orchard, grass cover	Cambisol (UKSO) Chalky, Silty Loam	7.2 ± 0.05	16.95 ± 0.4	38.7 ± 2.8	1.46 ± 0.2
2 - Exposed to soya and SNR	2019: Soybean (Siverka, SoyaUK) + *B. japonicum* SEMIA 5079 and *B. diazoefficiens* SEMIA 5080 (Legume Technologies) 2018: Winter wheat 2017: Oilseed rape 2016: Winter wheat 2015: Peas	Cambisol (UKSO) Clayey Loam to Sandy Loam	6.8 ± 0.04	14.96 ± 0.6	31.9 ± 6.2	1.61 ± 0.1
Welch two sample T-Test statistics	-	-	t = 5.1 df = 7.52 p < 0.01	t = 2.67, df = 7.35 p < 0.05	NS	NS

Table S2. Primers and PCR protocols used in this study.

Primer Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	PCR reaction mix	PCR programme	Reference
BOXAIR	CTACGGCAAGGCGACGCTGACG	N/A	• 15 µL GoTaq MM • 2.4 µL 10 mM BOX primer • 10.6 µL PCR grade water • 2 µL boil prep of single colony	94°C for 30s, 35 x (94°C for 10s, 50°C for 30s, 72°C for 30s), 72°C for 10min	Versalovic et al., 1994
16S	CCTACGGGAGGCAGCAG	ATTACCGCGGCTGCTGG	• 10 µL Roche Lightcycler 480 SYBR Green I Master Mix • 0.5 µL bovine serum albumin • 1 µL of each primer (10 µM) • 1 µL of DNA template • PCR grade water to 20 µL	95°C for 15 min, 40 x (95°C for 10s, 58°C for 10s, 72°C for 5s, Acquisition 82°C for 5s) 95°C continuous acquisition for melt curve	Muyzer et al., 1993
16S_mut	CCTACGGGAGGCACGTC	ATTACCGCGGCTGGACC	• 10 µL Roche Lightcycler 480 SYBR Green I Master Mix • 0.5 µL bovine serum albumin • 1 µL of each primer (10 µM) • 1 µL of DNA template • PCR grade water to 20 µL	95°C for 15 min, 40 x (95°C for 10s, 58°C for 10s, 72°C for 5s, Acquisition 82°C for 5s) 95°C continuous acquisition for melt curve	Modified from Daniell et al., 2012
rhiz_rpoB	GGYCGCGTSAARATGAACATGCG	GCRTTGATSAGRTCYTGYGGCATSAC	• 10 µL Roche Lightcycler 480 SYBR Green I Master Mix • 0.5 µL bovine serum albumin • 1 µL of each primer (10 µM) • 1 µL of DNA template • PCR grade water to 20 µL	95°C for 15 min, 50 x (95°C for 10s, 65°C for 10s, 72°C for 5s, Acquisition 86°C for 5s) 95°C continuous acquisition for melt curve	This study
BnodZ	TCGTCCTCGAGCAGGTTTCGGTTAA	CGAAGCCATAAGCGCTTGCGAGT	• 10 µL Roche Lightcycler 480 SYBR Green I Master Mix • 0.5 µL bovine serum albumin • 1 µL of each primer (10 µM) • 1 µL of DNA template • PCR grade water to 20 µL	95°C for 15 min, 45 x (95°C for 10s, 69°C for 10s, 72°C for 5s, Acquisition 85°C for 5s) 95°C continuous acquisition for melt curve	This study
SnodZ	TTGTACAATCGATATGTCC	ATCCTTGATTGGTTCAAAA	• 10 µL Roche Lightcycler 480 SYBR Green I Master Mix • 0.5 µL bovine serum albumin • 1 µL of each primer (10 µM) • 1 µL of DNA template • PCR grade water to 20 µL	95°C for 15 min, 50 x (95°C for 10s, 58°C for 10s, 72°C for 5s, Acquisition 87°C for 5s) 95°C continuous acquisition for melt curve	This study
16S rRNA (Amplicon sequencing primers with tags for secondary nested PCR highlighted in red)	ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNNNGTGCCAGCMGCCGCGGTAA	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGGACTACHVGGGTWTCTAAT	• 0.25 µL Phusion Taq polymerase • 5 µL HF Buffer • 0.5 µL 10 mM dNTPs • 1 µL DNA template • PCR grade water to 20 µL	95°C for 5 min, 25 x (94°C for 10s, 70.6°C for 30s, 72°C for 30s), 72°C for 5 min	Caporaso et al., 2011
rpoB (Amplicon sequencing primers with tags for secondary nested PCR highlighted in red)	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGGYCGCGTSAARATGAACATGCG	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTGCRTTGATSAGRTCYTGYGGCATSAC	• 0.25 µL Phusion Taq polymerase • 5 µL HF Buffer • 0.5 µL 10 mM dNTPs • 1 µL DNA template • PCR grade water to 20 µL	98 °C for 30s, 30 x (98°C for 10s, 72 °C for 10s), 72 °C for 5 min	This study

Supplementary Figures

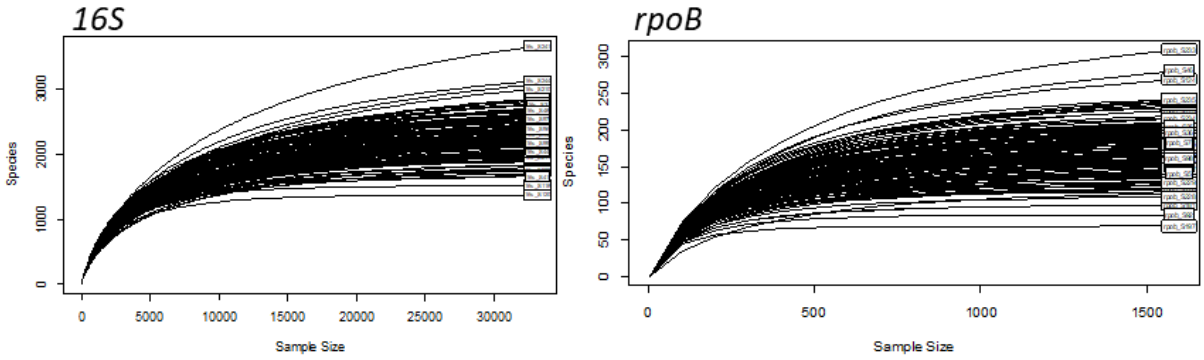


Figure S1. Rarefaction curves for 16S rRNA dataset to 33,175 sequences per sample and *rpoB* dataset to 1,537 sequences per sample. This lost the minimum amount of samples (10, including 3 DNA kit negatives) that did not meet the cut off for diversity analyses.

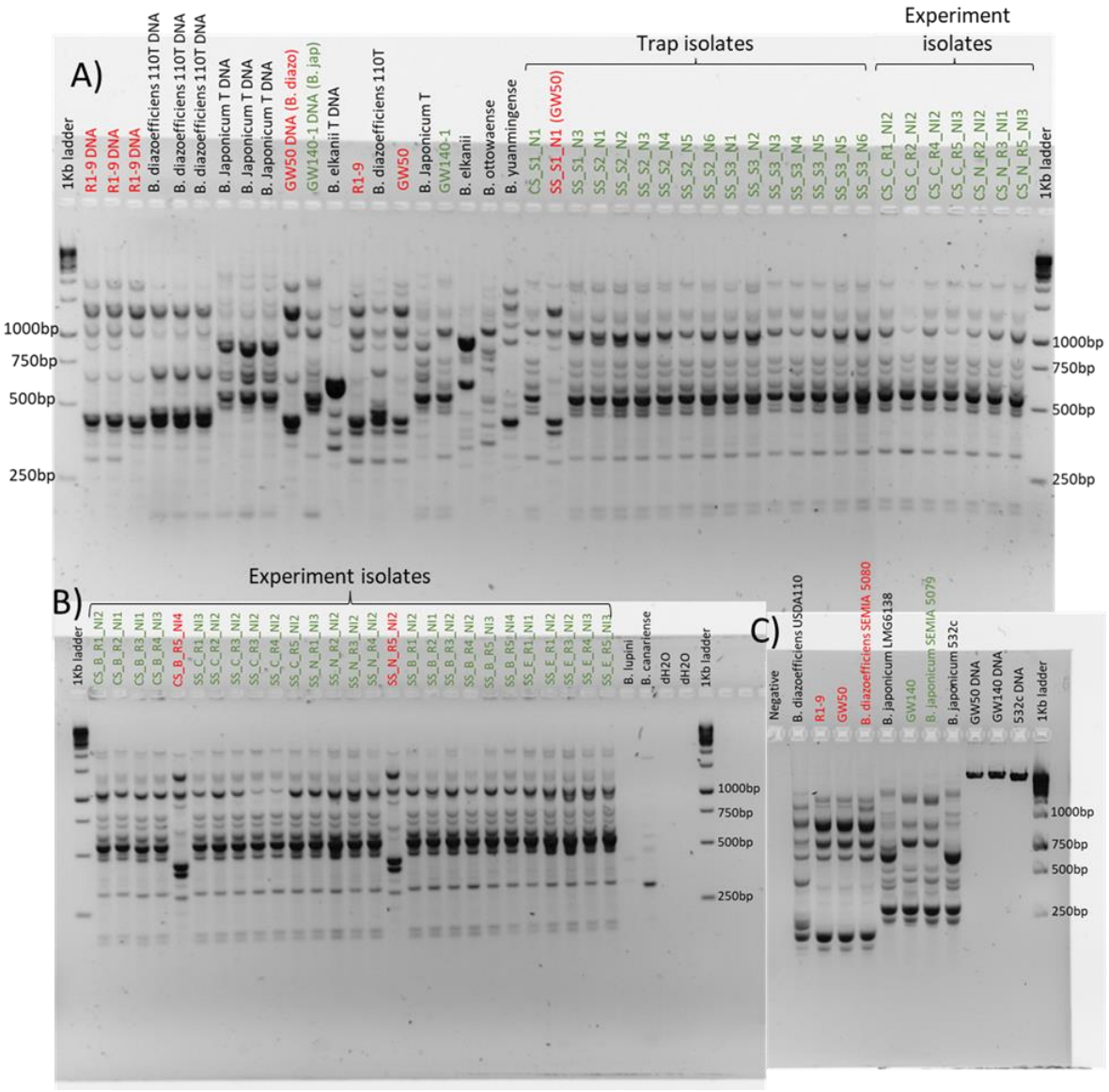


Figure S2. The majority of nodule isolates obtained from soils were *Bradyrhizobium japonicum* (highlighted in green). BOXPCR of slow growing (≥5 days) isolates extracted from the trap plants (A) and experiment nodules (A, B). No- soya isolates labelled "CS*", soya soil isolates labelled "SS*". R1-9 = *B. diazoefficiens* strain inoculated in this study, all those highlighted in red on gel A and B have a BOXPCR banding pattern that corresponds to *B. diazoefficiens* SEMIA 5080 as demonstrated in gel C. All those highlighted in green on gel A and B have a BOXPCR banding pattern that corresponds to *B. japonicum* SEMIA 5079 as demonstrated on gel C. GW50 (*B. diazoefficiens*) and GW140 (*B. japonicum)* are representative isolates that were extracted from the nodules of trap plants.

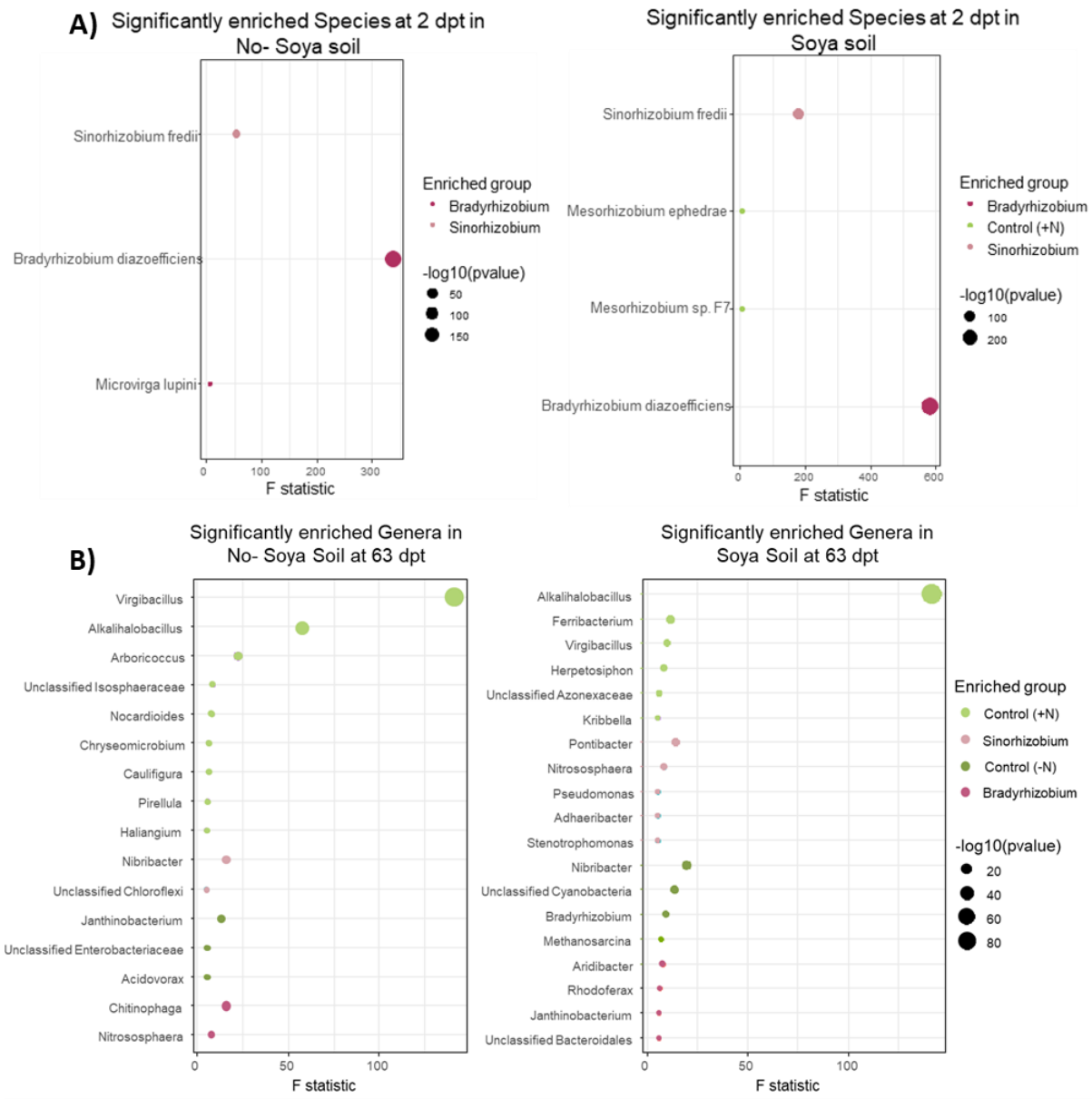


Figure S3. **A)** Dot plot of significantly enriched alphaproteobacterial species (*rpoB*) within the no-soya and soya soil microbiome under different inoculation treatments at 2 dpt. **B)** Dot plot of significantly enriched bacterial genera (16S rRNA) within the no- soya and soya soil microbiome under different inoculation treatments at 63 dpt. Size of the dot corresponds to the size of the p-value. Significantly enriched bacteria were assessed using the EdgeR test in the microbiomeMarker package (Cao *et al.*, 2022). A quasi-likelihood F- test was performed due to the stricter error rate control by accounting for the uncertainty in dispersion estimation, and a p- value cut off = 0.05 with a false discovery rate employed. As comparisons were between four inoculation treatments the function performed an ANOVA-like test to find markers which differed in any of the groups.

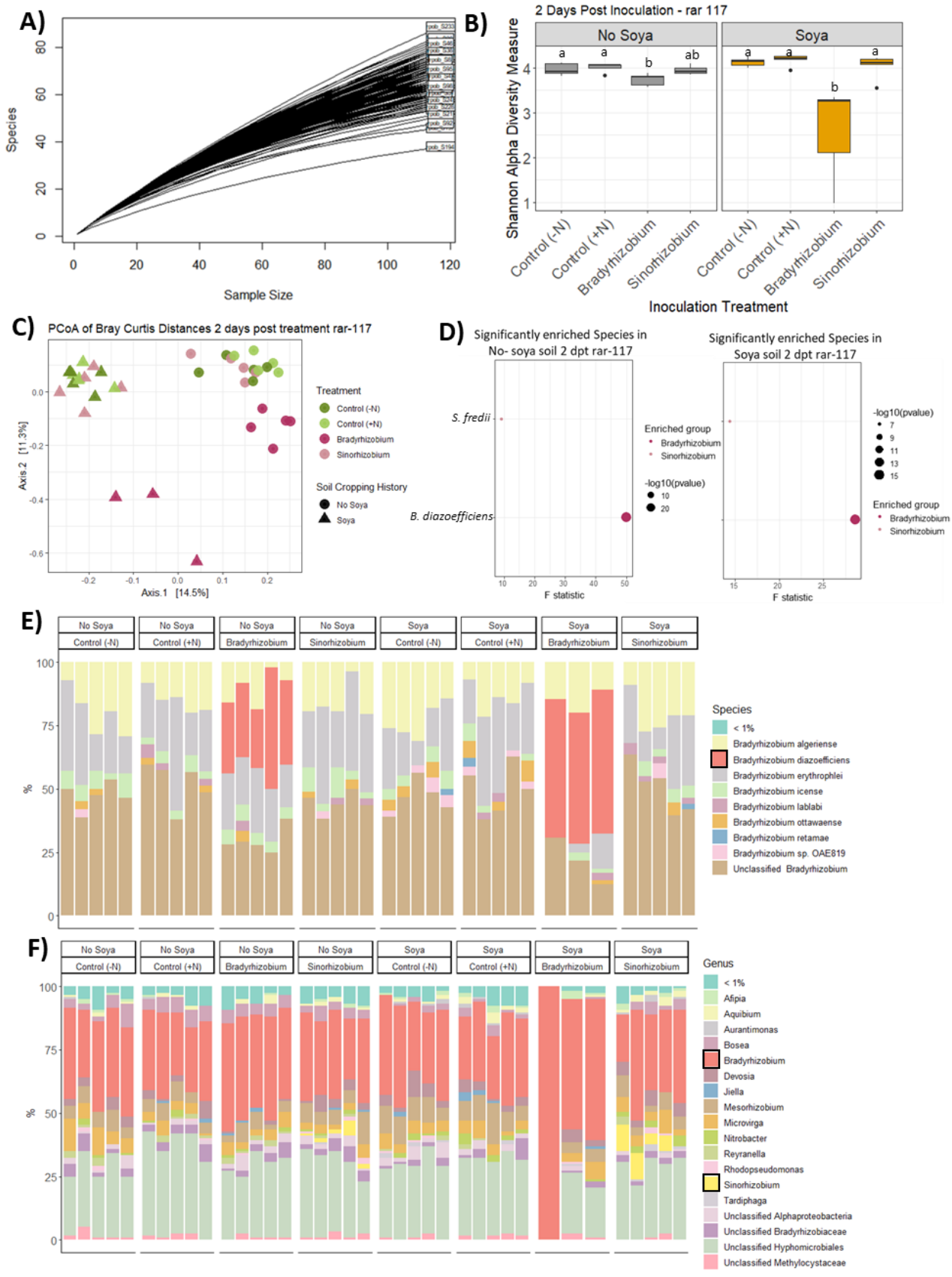
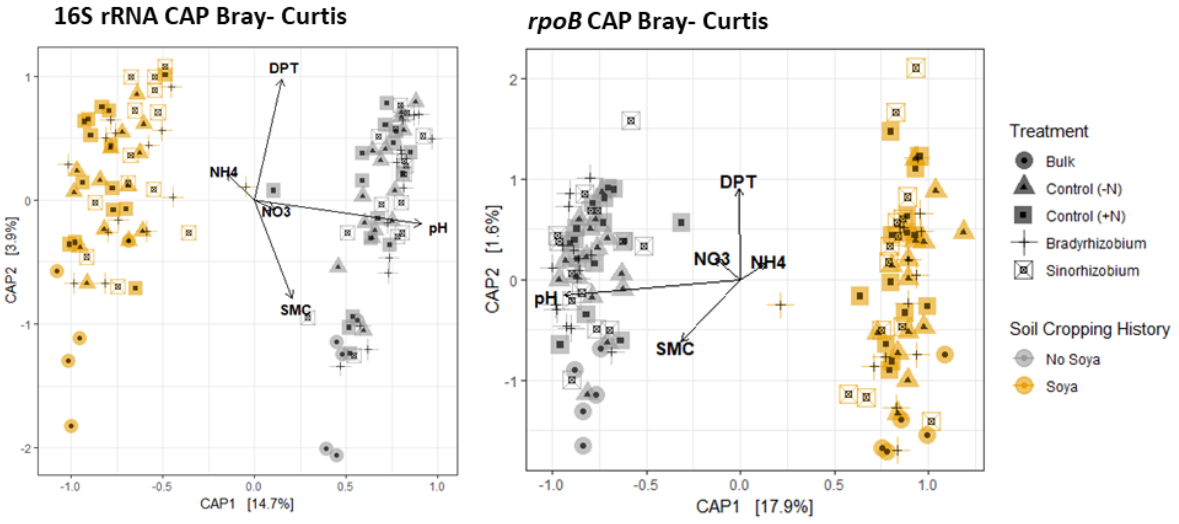


Figure S4. Diversity measures and significantly enriched taxa for *rpoB* alphaproteobacterial soil community rarefied to 117 sequences per sample to retain a third replicate in the *Bradyrhizobium*

treatment at 2dpt. This results in 1,023 taxa across 163 samples. **A)** Rarefaction curve to 117 sequences per sample. **B)** Shannon's Alpha Diversity Index at 2 days post inoculation, including the third replicate, *Bradyrhizobium* inoculation significantly reduces *rpoB* Shannon's diversity in both soil microbiomes. Different letters denote statistical significance by a Tukey's posthoc test ($p < 0.05$) within soil types. **C)** Principal Coordinates Analysis of Bray- Curtis Dissimilarity Matrix for alphaproteobacterial *rpoB* at 2 days post treatment, dark green = Control (-N), light green = Control (+N), dark pink = *Bradyrhizobium*, light pink = *Sinorhizobium*, circle = no soya soil, triangle = soya soil. **D)** Dot plot of significantly enriched species within the no- soya and soya soil microbiome under different inoculation treatments (EdgeR). Size of the dot corresponds to the size of the p- value. **E)** Relative abundance of *Bradyrhizobium* (*rpoB*) species at 2 dpt, black square highlights inoculant species**. F)** Relative abundance of *Alphaproteobacteria* genera (*rpoB*) at 2 dpt, black square highlights inoculant genera.



Response	DF	*16S* - Bray Curtis		*rpoB* - Bray Cutis	
		F	p- value	F	p- value
DPT (days post treatment)	1	5.7	0.001 ***	3.6	0.005 ***
pH	1	15.4	0.001 ***	19.5	0.001 ***
NO_{3-}	1	1.5	0.121	1.6	0.133
NH^{4+}	1	1.6	0.109	1	0.371
SMC (soil moisture content)	1	3	0.005 **	3.3	0.008 **
Residual DF		119		118	

Figure S5. Canonical Analysis of Principal coordinates (CAP) on Bray- Curtis distances for 16S rRNA and *rpoB* bacterial communities for timepoints 0, 22, 63, 84 dpt. Arrows represent effect size of days post treatment (DPT), soil pH, NO3-, NH4+ and soil moisture content (SMC) throughout the experiment. Grey = no-soya soil, yellow = soya soil, shape corresponds to inoculation treatment. Data presented excludes time point 2 dpt as soil traits were not collected for this time point. Table contains F statistics and p- values from permutation test on distance- based redundancy analysis (dbRDA - capscale), with marginal effects of terms tested, 999 permutations.

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Chapter 3: Assessing the impact of multi- species rhizobia inoculants on soybean productivity

3.1 Abstract

Inoculating legumes with nitrogen-fixing, root- nodulating symbionts (rhizobia) can increase plant productivity without the need for nitrogen fertilisation. However most inoculation studies have been conducted with single rhizobia strains, the impacts of inoculating a diverse interspecific rhizobial cohort on plant yields is less well researched. Competition between inoculant strains can occur and result in reduced plant benefits from the symbiosis. In Chapter 2 a reduction in seed biomass was observed when *Sinorhizobium fredii* was inoculated into soil already possessing compatible *Bradyrhizobium* symbionts. To investigate whether the combination of these inoculant strains leads to reduced plant biomass yields, a pot experiment in simple substrate (sand and vermiculite) was designed, with single rhizobia species and multi-species inoculant combinations for the ESG152 soybean cultivar. Nodule occupancy and plant biomass traits were assessed after 12 weeks. *S. fredii* inoculation resulted in a larger investment into the symbiosis and reduced plant biomass traits, however the multi-species inoculation (*Bradyrhizobium* and *Sinorhizobium)* resulted in a 55% increase in total biomass that was similar to all other *Bradyrhizobium* containing treatments. Nodule occupancy data suggests that when co-inoculated, *Bradyrhizobium* symbionts dominate. Multi-species treatments did not have reduced plant benefits, suggesting that competition between inoculant strains did not negatively affect plant growth in this experiment. Research in this area could benefit inoculant formulations for the improved efficiency of soya inoculant products.

3.2 Introduction

Legume hosts may benefit from receiving diverse rhizobia inoculants, particularly when introduced to a new environment. Soybean (*Glycine Max* L. Merr) growth in the UK is relatively new and requires seed inoculation to introduce compatible soya- nodulating rhizobia (SNR) to the soil microbiome (Coleman *et al*., 2021; Maluk *et al*., 2023). A widespread inoculation strategy for soybean globally is the co-inoculation with *Bradyrhizobium diaozefficiens* SEMIA 5080 and *Bradyrhizobium japonicum* SEMIA 5079, originating from strain selection programmes in Brazil (Hungria *et al*., 1996; Hungria and Vargas, 2000). Rationale behind this co-inoculation strategy is that the symbionts have different strengths, SEMIA 5079 is highly competitive for nodule occupancy and SEMIA 5080 is a more efficient N_2 fixer (Siqueira *et al*., 2014). Other soybean inoculant products may also contain other *B. diazoefficiens* and *B. japonicum* strains alongside *Bradyrhizobium elkanii* species (Chibeba *et al*., 2018; Thilakarathna and Raizada, 2017; Zilli *et al*., 2021), however no commercial inoculants currently contain *Sinorhizobium fredii* symbionts (Albareda *et al*., 2009). Including more diverse strains in inoculants could increase the range of environments where they could be effective, for example, *S. fredii* symbionts are adapted to alkaline soil environments (Tian *et al*., 2012) and are dominant symbionts in their native range of alkaline soil regions in China (Yang *et al*., 2018). However, competition between rhizobial symbionts can lead to reduced effectiveness of the symbiosis (Mendoza-Suárez *et al*., 2021), which may translate into plant yield costs (Rahman *et al*., 2023). Competition may occur within diverse inoculants that contain multiple compatible rhizobia, through direct mechanisms (e.g. competitive interference; Granato, Meiller-Legrand and Foster, 2019; Rahman *et al.*, 2023) or indirect mechanisms (resource exploitation; Stubbendieck and Straight, 2016).

Competitiveness of rhizobia will depend on legume host genotype, rhizobia genotype and the environmental context (G x G x E) interactions (Batstone, 2021; Batstone *et al*., 2023; Mendoza-Suárez *et al*., 2021). In agricultural environments, if compatible rhizobia are

already present in the soil, often inoculating crops with ‘elite’ rhizobia yields little to no yield improvements and inoculant strains get outcompeted for nodule occupancy by indigenous rhizobia. The indigenous, locally adapted rhizobia can be less efficient at N_2 fixation than elite strains, leading to reduced plant benefits than expected, contributing to a long known phenomena called the ‘rhizobia competition problem’ (Denton *et al*., 2002; Janice E Thies *et al*., 1991; Janice E. Thies *et al*., 1991; Triplett and Sadowsky, 1992). As legume hosts are exposed to a range of symbionts in the environment, they have evolved to discriminate between efficient and less efficient N_2 fixers, rewarding or sanctioning those strains, respectively (Denison and Kiers, 2004; Kiers *et al*., 2003; Regus *et al*., 2017; Westhoek *et al*., 2021, 2017). Inoculating a diverse consortia of compatible rhizobia may therefore increase the chances that at least one symbiont will be competitive in the introduced soil environment and hosts may be able to select the most beneficial symbiont in a given environment. Numerous inoculation studies have been carried out, often using single strain formulations, which prove beneficial in artificial environments, but have varied effects in soybean field settings (Thilakarathna and Raizada, 2017). However the impact of inoculating a diverse consortia of rhizobia species on legume productivity is less well studied and has led to varied findings, ranging from a negative impact on plant biomass in *Acacia* species (Barrett *et al*., 2015) to neutral impacts in clover species (Fields *et al*., 2021).

The results in Chapter 2 suggested that *S. fredii* may be able to outcompete *B. japonicum* SEMIA 5079 for nodule occupancy in certain soil environments, but when a larger population of SNR were already present (*i.e.* in previously inoculated soya soil), inoculation with *S. fredii* decreased soybean seed biomass compared to uninoculated plants. Potentially, these reduced plant benefits could be due to competitive interactions between the previously introduced *Bradyrhizobium* symbionts (SEMIA 5079 and SEMIA 5080) and *S. fredii* inoculant either in the soil microbiome or *in planta*. To investigate this further, the complexity of the soil microbiome was removed and a pot experiment in a simple substrate media (sterilised sand and vermiculite) was devised to assess plant biomass traits under

single- species or multi- species combinations of the inoculants used in Chapter 2. Inoculation with *S. fredii* is expected to result in lower plant biomass yields and higher investment into the symbiosis and inoculation with *Bradyrhizobium* and *Sinorhizobium* may result in lower plant biomass traits due to competition for nodule occupancy by *S. fredii*. Research in this area could improve inoculant formulations for soybean productivity.

3.3 Methods

Plant and bacteria growth conditions

Inoculant strains used in Chapter 2 (*B. diazoefficiens* R1-9 and *S. fredii* 495), along with rhizobia isolated from the Kent soybean field soil (*B. diazoefficiens* GW50 and *B. japonicum* GW140) were used to design single strain and multi- strain inoculation treatments for this experiment (see Table 1). *Bradyrhizobium* species were grown in modified yeast mannitol broth for 5 days and *S. fredii* was grown in tryptone yeast broth for 3 days before inoculant formulation, where bacteria were standardised to 10^8 CFU/ mL^{-1} (Howieson and Dilworth, 2016). Multi-strain inoculants were made up of equal volumes of standardised (10^8 CFU/ mL^{-1}) bacterial cultures and were resuspended in sterilised rhizobia wash buffer (sterilised 10 mM MgSO4 and 0.01% Tween 40) for inoculation. The same early maturing soybean cultivar (ESG152, Euralis, France) used in Chapter 2 was used in this experiment. Plants were grown in greenhouse conditions with 16 h day at 25 °C and 15 °C at night. Plants were fed 20 mL N- free CRS solution weekly for the first 5 weeks, then 40 mL for the remaining 7 weeks. The N control was supplemented with 2.2g L^{-1} NH_4NO_3 (equivalent to 30 kg ha^{-1}) each week.

Experimental design

A pot experiment was designed to test the impact of inoculating single SNR species and multi-SNR species on soybean plant biomass traits and nodule occupancy (Table1). The current dual inoculation strategy (GW140/ SEMIA 5079 and GW50/ SEMIA 5080) was

combined with the novel symbiont *S. fredii* or the more 'adapted' *B. diazoefficiens* R1-9 isolated from soils in Dundee, Scotland (Maluk *et al*., 2023), alongside their respective single strain inoculation treatments and two controls, one supplied with N fertiliser and one without. Soybean seeds were sterilised (shaking in 2.5% NaOCl for 10 minutes then washed with sterile water six times) and left to germinate on 0.5% agar plates at 25 ℃ for three days. Then, seedlings were planted into twice autoclaved sand: vermiculite at 4:1 in 1 L tricorn pots and each seedling was inoculated with 1 mL of standardised bacterial or mock inoculant. Inoculants were spot- plated at a range of dilutions on yeast mannitol agar plates to check for cross-contamination (Howieson and Dilworth, 2016). Colonies were visually inspected (*Sinorhizobium* colonies grow by 3 d, *Bradyrhizobium* take 5 - 7 d) and subject to the multiplex PCR described below for rhizobia typing, which yielded all expected strains in the treatments. Six biological replicates were planted over two days, which were three days apart (block 1 = R1 - R3 on day 1, block 2 = R4 - R6 on day 4), plants were harvested within their blocks at 12 weeks. At harvest, aboveground biomass was separated from belowground biomass and dried at 80 °C for 48 hours. Root nodules were removed and counted, 10 nodules per plant replicate were pooled, sterilised (1 min in 70% EtOH, 3 mins 2.5% NaOCl, 6 sterile dH_2O washes) and crushed in 750 µL rhizobia wash buffer. Serial dilutions of 10^{-5} - 10^{-7} were plated on yeast mannitol agar plates (Howieson and Dilworth, 2016). After 5 days of growth, 12 colonies along a transect line in the middle of the agar plates were picked into 50 µL of nuclease free water, within which they were boiled (95 °C for 5 min then centrifuged at 4000 rpm for 1 minute) and then used for rhizobia typing by multiplex PCR. The remaining root nodules and roots were dried at 80 °C for 48 hours, then weighed for plant biomass.

Table 1. A list of treatments, strain combinations, their isolation history and justification for use in this experiment.

Inoculation treatment	Rhizobia strains	History and justification
Control (-N)	Sterile buffer solution	N/A
Control (+N)	Sterile buffer solution + N (30kg/ ha) supplemented weekly	N/A
Bd_R1-9	Rizoliq1-9 *Bradyrhizobium diazoefficiens*	Isolated from soybean (Commandor, Euralis) nodules post inoculation field trial in Dundee, Scotland (Maluk et al 2023). Used as inoculant strain in Chapter 2.
Bd_GW50	GW50 *Bradyrhizobium diazoefficiens SEMIA 5080 variant*	Isolated from soybean (ESG152, Euralis) nodules grown in soil sampled from Kent, England, that had previously been inoculated and cropped with soybean (SoyaUK; Described in Chapter 2).
Bj	GW140 *Bradyrhizobium japonicum SEMIA 5079 variant*	Isolated from soybean (ESG152, Euralis) nodules grown in soil sampled from Kent, England, that had previously been inoculated and cropped with soybean (SoyaUK; Described in Chapter 2).
Sf	495 soya 32-2 *Sinorhizobium fredii*	Isolated from soybean nodules grown as trap plants in soil from the alkaline (Karst limestone) soybean-growing areas in China. Used as inoculant strain in Chapter 2.
Bd_R1-9 x Bd_GW50 x Bj	R1-9, GW50, GW140 *B. diazoefficiens* (Dundee isolate), *B. diazoefficiens* (Kent isolate) and *B. japonicum*	This treatment mimics the rhizobia present in the *Bradyrhizobium* treatment soya soil from chapter 2.
Sf x Bd_GW50 x Bj	495, GW50, GW140 *S. fredii, B. diazoefficiens and B. japonicum*	This treatment mimics the rhizobia present in the *Sinorhizobium* treatment soya soil from chapter 2.
Bd_GW50 x Bj	GW50, GW140 *B. diazoefficiens* (Kent isolate) *and B. japonicum*	This treatment mimics the previous inoculant rhizobia present in the soya soil in chapter 2.
Bd_R1-9 x Bj	R1-9, GW140 *B. diazoefficiens* (Dundee isolate) *and B. japonicum*	This treatment was included to test the efficacy of the Scottish *B. diazoefficiens* isolate with the competitive *B. japonicum* strain to see if it differed from the Kent isolate (GW50).

Multiplex PCR for identifying soybean rhizobia

A multiplex PCR test was designed to identify nodule colony isolates by targeting the RNA polymerase B (*rpoB)* core gene. Primers were designed by eye based on an alignment of the *rpoB* gene extracted from 45 rhizobia species spanning *Alphaproteobacteria*. Primer specificity was tested on panel of *Bradyrhizobium* and *Sinorhizobium* strains including: *B. diazoefficiens* R1-9, *B. diazoefficiens* GW50, *B. diazoefficiens* USDA110^{T}, *B. japonicum* GW140, *B. japonicum* USDA6^{T}, *B. elkanii* USDA76^{T}, *B. ottowaense* HAMBI3284^{T}, *B. yuanmingense* LMG21827^{T}, *S. fredii* HH103, *S. meliloti*^{T} LMG6133, *S. medicae*^{T} LMG 6133. A combination of 5 primers was used to create a different banding pattern for *B. diazoefficiens, B. japonicum* and *S. fredii* (see Table 2). The primer set Bjd_rpob1 (forward and reverse) targets both the *B. diazoefficiens* and *B. japonicum rpoB* gene resulting in a 137bp product. An additional forward primer Bd_rpob2 only targets *B. diazoefficiens* strains and combined with the reverse primer of Bjd_rpob1 results in a 900 bp product. This results in two bands for a *B. diazoefficiens* strain (900 bp and 137 bp) and only one band for *B. japonicum* strains (137 bp). The primer set Sf_rpob2 is also included in the PCR reaction mix and results in a 215 bp product for an *S. fredii* strain (see Image 1). All primers used proved specific to *B. japonicum, B. diazoefficiens* and *S. fredii* species as intended.

Table 2. A list of primers and PCR conditions used in this study.

Primer Name	Forward (5' – 3')	Reverse (5' – 3')	Target	PCR conditions
Bjd_rpob1	GAAGGCGCTGCGSCTGT	TGCTCGTTGAGGGCCTTCAT	*Bradyrhizobium diazoefficiens* *Bradyrhizobium japonicum*	Per reaction: • 12.5 µL GoTaq Polymerase • 8 µL Nuclease free water • 1 µL Bjd_rpob1 • 0.5 µL Bd_rpob2 • 1 µL Sf_rpob2 • 2 µL Boil prepped colony 94 for 5 min, [94 for 10s, 65 for 30s, 72 for 30s] x 30, 72 for 5 min Run on a 2% gel at 80V for 1hr
Bd_rpob2	GATGGTCGACGAACCCCAG	N/A	*Bradyrhizobium diazoefficiens*	
Sf_rpob2	CCTATAAGGCCGGAGCTGAC	CTTCAGACCTGCCTGCTCAA	*Sinorhizobium fredii*	

Data Analysis

Data were analysed on R (v4.1.3) with R studio (R Studio Team, 2020). To assess whether symbionts were at equal proportions for nodule occupancy, which could indicate no significant competition between strains or no influence of other external factors such as legume host preference, Chi- square tests were performed on raw counts. To give an estimate of how much plants were investing in the symbiosis, nodule investment was calculated as nodule biomass divided by aboveground biomass (Rahman *et al*., 2023). Soybean growth response to inoculation was estimated by dividing the aboveground biomass values of inoculated plants by the aboveground biomass values of uninoculated, unfertilised control plants (Rahman *et al*., 2023). Harvest index gives a proxy of how much plants invested into their seeds and was calculated as a percentage by dividing seed biomass by total biomass. Linear models were constructed to test the impact of inoculation treatment and planting block on soybean biomass traits, assumptions of the models were checked and models were subjected to an ANOVA. Where necessary data were transformed before statistical tests to meet the assumptions. Post-hoc comparisons (Tukey HSD tests) were performed to determine which treatments significantly differed from each other. Statistics were performed on the dataset with contaminated replicates removed, this

reduced replication to 2 in the Sf treatment, therefore nodule investment and growth response to inoculation for the whole dataset, including contaminated replicates is also presented. Contrasts between coefficients within linear models were used to compare inoculation treatments and are provided to support treatment effects (*t* and *p* - values).

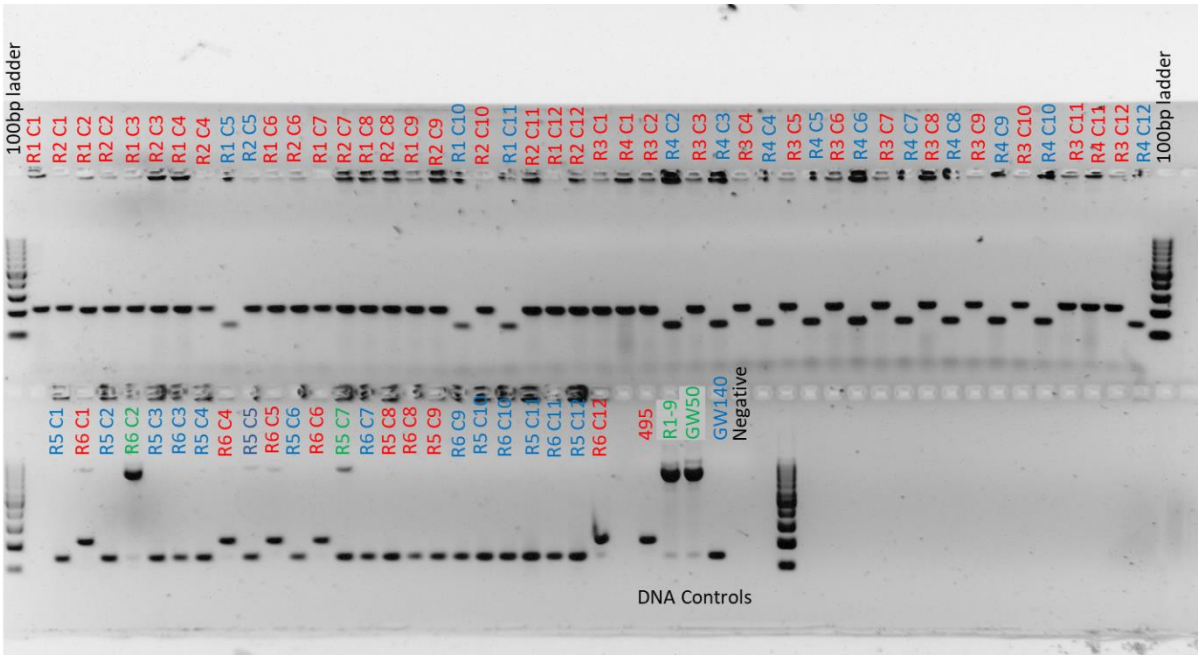

Image 1. Example gel image of multiplex PCR for rhizobia typing. Ladder range is from 100 – 1000 bp increasing in 100 bp increments. Controls with rhizobia strain DNA and PCR negative located at bottom right of gel. Red = *S. fredii* (215 bp product), blue = *B. japonicum* (137 bp product), green = *B. diazoefficiens* (900 and faint 137 bp product). Samples are from Sf treatment, 12 colonies for each replicate.

3.4 Results

Strain nodule occupancy

All uninoculated controls, with and without N were non-nodulated in this trial. However, when assessing the symbiont proportions in the nodules of the inoculated treatments, some plant

replicates had become cross-contaminated with inoculant rhizobia that were not present in the starting inoculant (Figure 1). Since inoculants at the start of the experiment were checked for correct strain presence, this suggests they became cross-contaminated in the greenhouse chamber throughout the growing period. Cross- contamination was found in four replicates in the Sf treatment, with only two replicates remaining nodulated by *S. fredii* alone. In the Bd_GW50 x Bj treatment, one replicate had *S. fredii* present. As this study aims to link biomass phenotypes to rhizobia symbiont strains, the cross- contaminated replicates were removed from the soybean biomass analysis. However, it is interesting that despite *S. fredii* being inoculated at large populations in the Sf treatment, *Bradyrhizobium* symbionts were still able to colonise and become the most dominant symbiont in some plant replicates (specifically the *B. japonicum* strain). In the Sf x Bd_GW50 x GW140 treatment, *Bradyrhizobium* symbionts dominate nodule occupancy and all three symbionts are not at equal proportions as would be expected by chance (χ^2 = 28.6, *d.f.* = 2, $p < 0.001$, see Table 3). All multi-strain treatments had unequal symbiont proportions apart from the Bd_R1-9 x Bj treatment (Table 3), where Bd_R19 was at a higher abundance and at near equal counts to Bj (34 and 38 respectively).

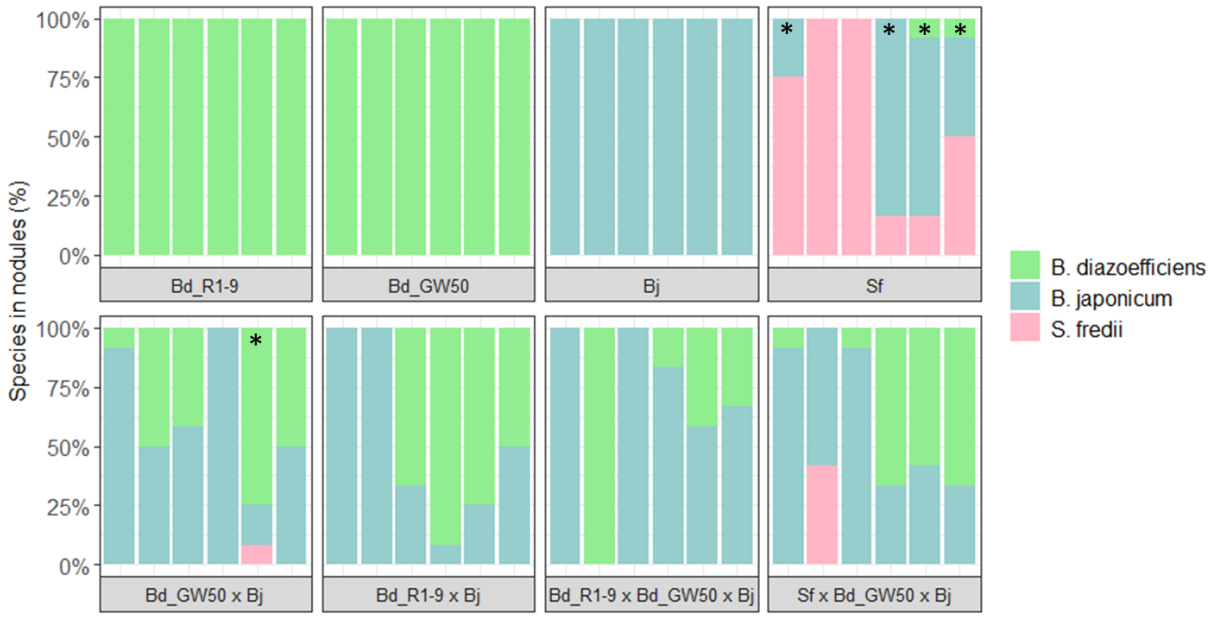

Figure 1. Percentage of soya- nodulating rhizobia species in nodules within different inoculation treatments. Black stars denote replicates that were cross- contaminated. Green = *B. diazoefficiens*, blue = *B. japonicum* and pink = *S. fredii*.

Table 3. Results of nodule occupancy raw counts and Chi-square tests.

Treatment	B. diazoefficiens	B. japonicum	S. fredii	Chi – square
Bd_R1-9	72	-	-	N/A
Bd_GW50	72	-	-	N/A
Bj	-	72	-	N/A
Sf	2	27	43	χ^2 = 35.6, df = 2, p < 0.001
Bd_R1-9 x Bd_GW50 x Bj	23	49	-	χ^2 = 9.4, df = 1, p < 0.01
Sf x Bd_GW50 x Bj	25	42	5	χ^2 = 28.6, df = 2, p < 0.001
Bd_GW50 x Bj	27	44	1	χ^2 = 39.1, df = 2, p < 0.001
Bd_R1-9 x Bj	34	38	-	NS

Bradyrhizobium symbionts result in higher plant productivity for less investment in nodulation

When investigating plant biomass traits of the uncontaminated replicates, inoculation treatment had a significant effect on total (ANOVA: $F_9 = 30.4$, $p < 0.001$), shoot (ANOVA: $F_9 = 11.2$, $p < 0.001$) root (ANOVA: $F_9 = 15.7$, $p < 0.001$) and seed biomass (ANOVA: $F_9 = 9.1$, $p < 0.001$), with no significant effect of planting block (Figure 2). All inoculation treatments containing *Bradyrhizobium* strains significantly increased total biomass compared to the Sf treatment ($t = 3.8$, $p < 0.001$). The N- fertilised control resulted in larger root biomass than all inoculated treatments, yielded similar total and shoot biomass to the inoculated treatments, but gave lower seed biomass similar to the uninoculated, non- fertilised control (Figure 2). When comparing the Sf treatment to the *Sinorhizobium* and *Bradyrhizobium* treatment (Sf x

Bd_GW50 x Bj), there is an average increase of 55% in total plant biomass for the multi-species treatment (t = 3.6 p < 0.001). Whereas, the *Bradyrhizobium* treatment without *Sinorhizobium* (Bd_GW50 x Bj) yields a similar total biomass to the three- way (Sf x Bd_GW50 x Bj) multi- strain treatment. Inoculation treatment also had a significant effect on growth response to inoculation (ANOVA: F_7 = 5.2, p < 0.001) and harvest index (ANOVA: F_9 = 8.2, p < 0.001). Plants in the Sf treatment had significantly lower growth response to inoculation, but, harvest index indicated that plants in the Sf treatment were still investing around 25% of aboveground biomass into seed production, which was statistically similar to all other inoculated treatments (Figure 3).

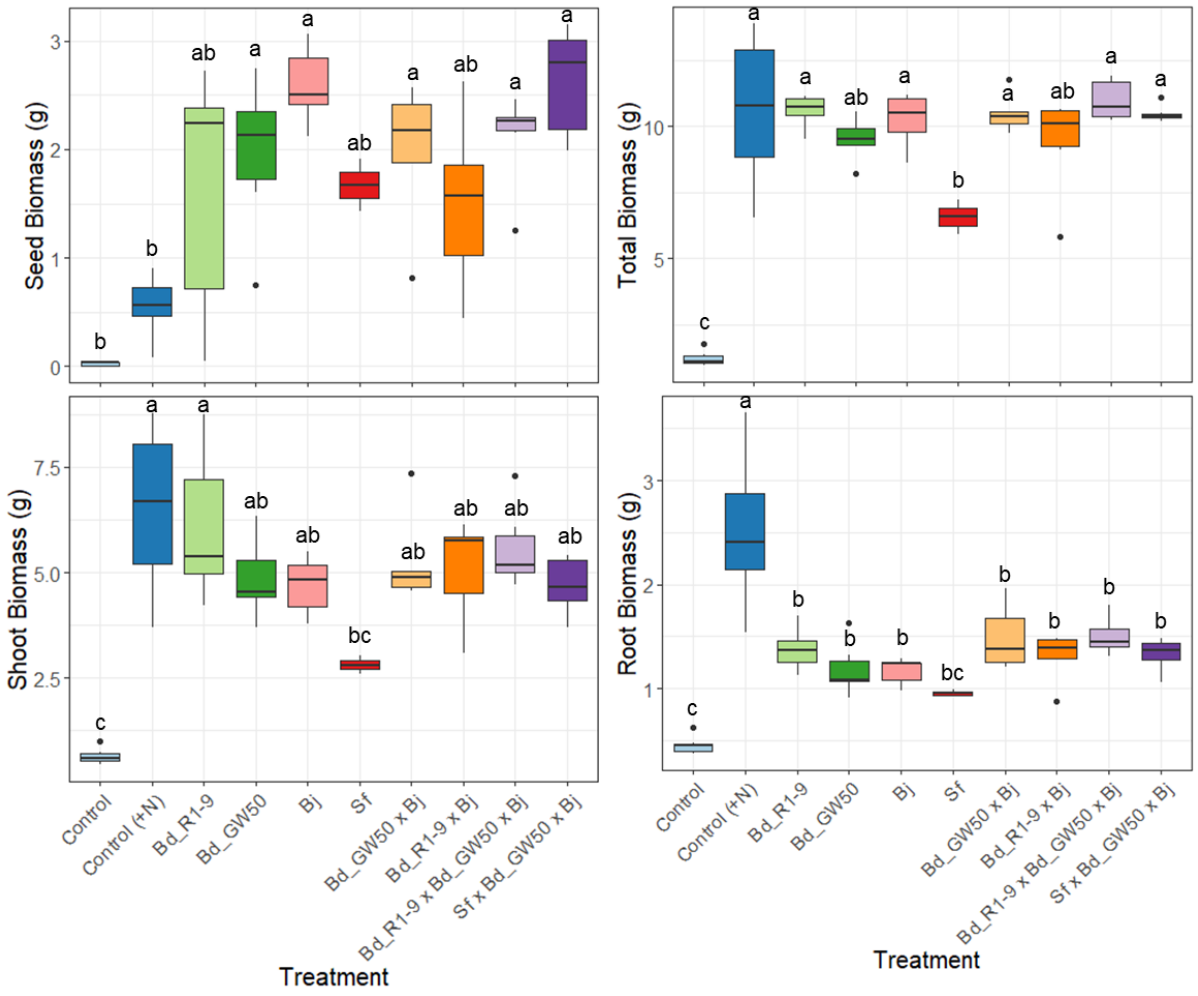

Figure 2. Soybean seed, total, root and shoot biomass traits under different inoculation treatments. Different letters denote statistical significance from Tukey HSD post- hoc tests (p < 0.05).

Inoculation treatment had a significant impact on nodule numbers (ANOVA: $F_7 = 9.5$, $p < 0.001$) and nodule biomass (ANOVA: $F_7 = 28.7$, $p < 0.001$). *Sinorhizobium fredii* inoculation induced significantly more nodules, and therefore yielded higher nodule biomass (Figure 4). Planting block 1 had significantly larger nodule biomass overall (ANOVA: $F_1 = 5.7$, $p < 0.05$), this was across treatments and thus did not mask treatment effects. Nodule investment was significantly impacted by inoculation treatment (ANOVA: $F_7 = 55.3$, $p < 0.001$). *Sinorhizobium fredii* inoculated plants invested significantly more into nodule production than all treatments containing *Bradyrhizobium* symbionts ($t = 19.6$, $p < 0.001$).

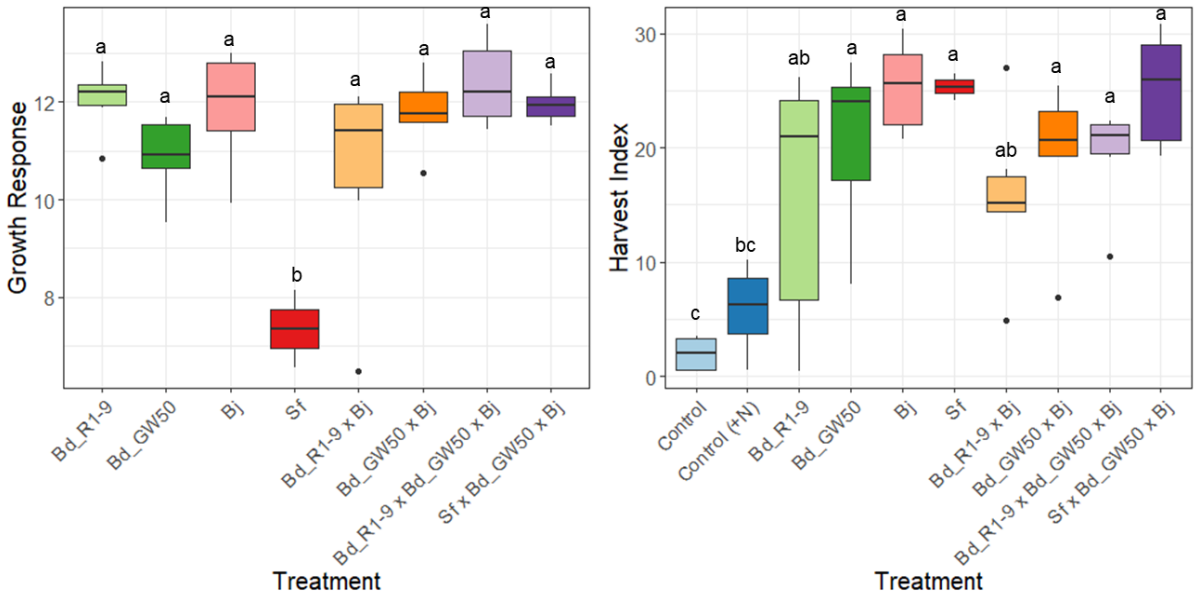

Figure 3. Soybean growth response to inoculation and harvest index (%) calculated as the percentage invested in seeds. Different letters denote statistical significance from Tukey post- hoc tests ($p < 0.05$).

As cross- contamination resulted in the removal of 4 out of 6 samples in the Sf treatment, the full dataset retaining the contaminated samples was analysed for growth traits (Figure 5). Inoculation treatment had a significant effect on nodule investment (ANOVA: F_7 = 7.7, $p < 0.001$), where there was also a significant effect of planting block across treatments (ANOVA: F_1 = 6.7, $p < 0.05$). Including the contaminated samples, nodule investment was still significantly higher in the Sf treatment (Figure 5). Similarly, Sf inoculation resulted in a significantly reduced growth response compared to all other inoculated treatments (ANOVA: F_7 = 9.3, $p < 0.001$), highlighting that plant replicates that contained *S. fredii* symbionts invested more into the symbiosis but received less growth benefit from it. Additionally, *S. fredii* inoculated plants that became cross contaminated with *Bradyrhizobium* symbionts (highlighted in red Figure 5) had a trend towards reduced nodule investment and these samples showed a more varied growth response to inoculation.

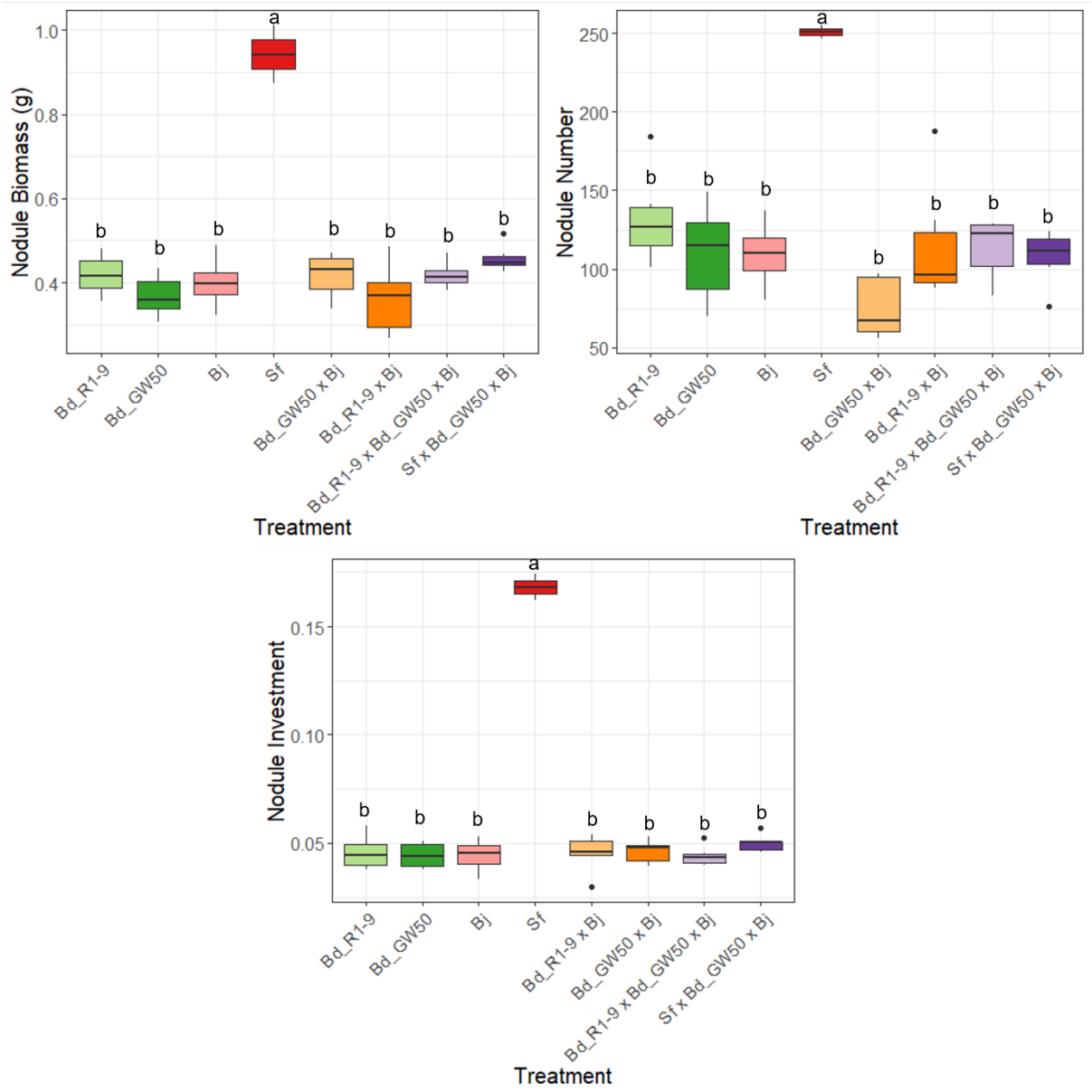

Figure 4. Symbiosis traits, nodule biomass, nodule numbers and nodule investment under different inoculation treatments. Different letters denote statistical significance from Tukey post- hoc tests ($p < 0.05$).

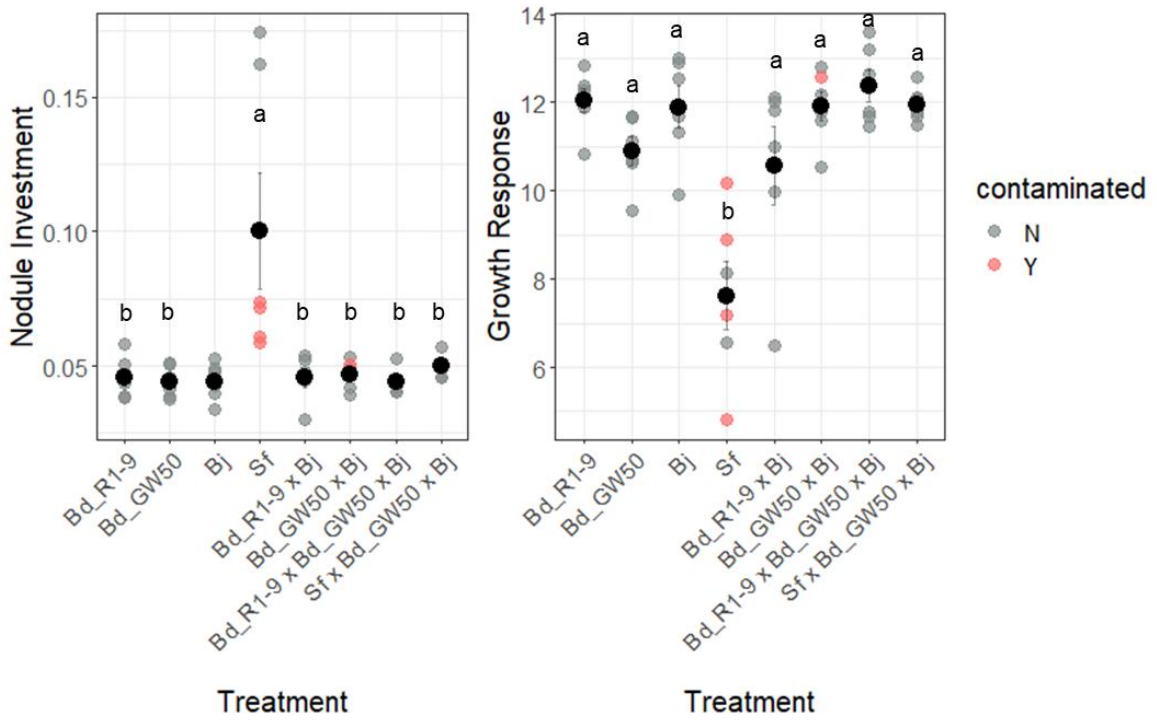

Figure 5. Nodule investment and growth response to inoculation under different inoculation treatments for the full dataset. Grey points = replicates that were not cross contaminated, red = cross- contaminated, black = mean, error bars = standard error (*n* = 6). Different letters denote statistical significance from Tukey post- hoc tests ($p < 0.05$).

3.5 Discussion

This experiment was conducted to investigate the impacts of inoculating multiple compatible rhizobia species on soybean plant biomass traits. In Chapter 2, a decrease in soybean seed biomass was observed when *S. fredii* was inoculated into soil already containing compatible *Bradyrhizobium* species. One potential reason for this could be that competition between rhizobia strains may be impacting the benefits received by host plants (Rahman *et al*., 2023). Therefore, this experiment looked to identify whether reduced plant benefits could be attributed to competition between symbionts or whether it's due to symbiont performance and occurrence within nodules. In contrast to the hypothesis, the multi-species treatment (Sf

x Bd_GW50 x Bj) yielded consistently high plant productivity, similar to other inoculation treatments apart from Sf. The results suggest that the symbiont *S. fredii* induces a much higher investment in the symbiosis, for a lower return in plant biomass for the early maturing soybean cv. ESG152 in this sterile pot environment. When provided with multiple compatible symbionts, including *S. fredii* and *Bradyrhizobium* species, host plant nodules were occupied by *Bradyrhizobium* symbionts over *S. fredii,* resulting in significantly larger total plant biomass and reduced investment to nodulation compared to *S. fredii* alone treatment.

Recent research has found competition between compatible rhizobia can impact the benefits received by a host legume, with competition between highly beneficial rhizobia strains reducing plant benefits the most (Rahman *et al*., 2023). Rahman *et al*., (2023) compared single strain and pairwise inoculant combinations of *Bradyrhizobium* symbionts with varying N_2 fixation capability, on *Acmispon strigosus* biomass, finding co-inoculated plants received less growth benefits in comparison to single strain inoculations. In all instances where highly efficient N_2 fixers were competing against lower quality partners, the high quality symbionts dominated nodule occupancies, which is reflected in this study where *Bradyrhizobium* symbionts largely outcompeted *S. fredii* in the Sf x Bd_50 x Bj treatment. Effective host sanctions may also play a role here, limiting the amount of carbon to less efficient nodules, reducing their occurrence and thus population growth within nodules (Kiers *et al*., 2003; Westhoek *et al*., 2021, 2017). In addition, the cross- contamination in the Sf treatment is interesting, because despite being inoculated at high population densities with *S. fredii*, when given the opportunity, plants were colonised by *Bradyrhizobium* symbionts over *S. fredii*. *Sinorhizobium* occupation of the two replicates that remained uncontaminated in the Sf treatment supports the previous finding that legume hosts will tolerate intermediate fixing strains if no better strains are available (Westhoek *et al*., 2021). However, contrary to the results found in this experiment, Rahman *et al* (2023) also find a greater reduction in host benefits when the most efficient strains are co-inoculated and had reduced nodule numbers, potentially attributing this to competitive interference amongst strains, due to their

reduced growth when co-inoculated in liquid media. It is important to note, in this soybean-rhizobia study system, a decrease in nodule number and biomass was actually indicative of improved biomass traits, further highlighting how rhizobia and plant host fitness traits are often not aligned (Burghardt *et al*., 2018; Burghardt and diCenzo, 2023).

In this study, single and multi-strain inoculation treatments containing *Bradyrhizobium* species yielded similar growth responses. The single inoculation treatments Bd_GW50 (*B. diazoefficiens* SEMIA 5080) and Bj (*B. japonicum* SEMIA 5079) yielded similar plant biomass to each other, to their combined treatment Bd_GW50 x Bj and even to the Sf x Bd_GW50 x Bj treatment, suggesting that they may be able to compensate for the presence of lower quality partners to some extent. This is consistent with results in clover, whereby the performance of a diverse intra-specific *R. leguminosarum* inoculum was best predicted by the yield benefits provided by the best performing member of that inoculum (Fields *et al*., 2021). The multi-strain treatment Bd_GW50 x Bj simulates the widely employed inoculation strategy for soybean (*B. diazoefficiens* SEMIA 5080 and *B. japonicum* SEMIA 5079). SEMIA 5080 and SEMIA 5079 co-inoculation is used due the reported higher N_2 fixation of SEMIA 5080, but higher competitive nodulation of SEMIA 5079 (Siqueira *et al*., 2014), which is indeed reflected in the nodule occupancy data where *B. japonicum* occurrence is 1.6 times that of *B. diazoefficiens* in the Bd_GW50 x Bj treatment. However, plant biomass data in this experiment shows no significant differences between the two single inoculation treatments Bd_GW50 and Bj, potentially suggesting that translation into plant biomass benefits may be evenly matched.The Bd_R19 x Bj treatment was the only treatment where we observed equal symbiont proportions as expected by chance, suggesting that the *B. diazoefficiens* strain isolated in Scotland, Bd_R1-9, may be able to match the *B. japonicum* strain for nodule occupancy in this relatively simple environment, although this treatment lead to more variable impacts on plant biomass.

This experiment was conducted in a sterilised sand and vermiculite potting mixture, where the complexity of the soil microbial community and by extension other ecological interactions within the microbial community were removed. However, it is known that other, non- rhizobial members of microbial communities can impact the growth and success of rhizobial symbionts in the rhizosphere. For example, *Bacillus cereus* isolates from saline-alkaline soil promoted *S. fredii* CCBAU45436 but inhibited *B. diazoefficiens* USDA110 during soybean symbiosis (Han *et al*., 2020). Non-rhizobial endophytes within legume nodules have been found to increase with time and in the *Medicago sativa* - *Sinorhizobium meliloti* symbiosis, NREs were found to produce antimicrobials that inhibited the growth of the rhizobial symbiont (Hansen *et al*., 2020). Thus, a combination of edaphic factors, biotic interactions both between compatible rhizobia and within the wider microbial community can lead to different outcomes for plant productivity.

3.6 Conclusion

Competition between rhizobial symbionts can influence the plant benefits received from the symbiosis. However, in this experiment, *S. fredii* induced a larger investment into the symbiosis for lower plant biomass and the multi-species inoculated treatments resulted in increased plant yields. This was due to a higher colonisation by *Bradyrhizobium* symbionts that dominated nodule occupancy in co-inoculated treatments. However, the harvest index of *S. fredii* inoculated plants is similar to *Bradyrhizobium* inoculated plants, indicating that the same proportion of plant resources are allocated into seed biomass. As many inoculation studies only assess early plant vegetative growth, this highlights the importance of growing inoculated plants to seed production in order to observe effects on this agronomically important trait. Many new methods have aimed at quantifying both N_2 fixation and competitiveness for nodulation simultaneously in environmental contexts (Burghardt *et al*., 2018; Mendoza-Suárez *et al*., 2020). If such technologies can be deployed in an agricultural setting, they will be a useful tool in the development of tailored inoculants.

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Chapter 4: Assessing beneficial plant growth promoting rhizobacteria in combination with *Bradyrhizobium* for temperate soybean performance

4.1 Abstract

The use of microbial inoculants in agriculture is expected to increase as the demand for more sustainable agricultural practices grows. Applying nitrogen-fixing symbionts, known as rhizobia, to legume crops is a well-known practice for supplying biologically fixed nitrogen in place of fertilisation. The benefits of co-inoculating other plant growth-promoting rhizobacteria (PGPR) with compatible rhizobial symbionts has been shown to improve plant biomass and other plant physiological characteristics. Soybean (*Glycine Max* L. Merr) is a recently introduced crop to the UK, yet there is little research investigating the effects of rhizobia and PGPR co-inoculation on temperate adapted varieties. In this study, the effects of co-inoculating a panel of PGPR with rhizobia were assessed for a temperate soybean cultivar widely grown in the UK, with plant biomass and nitrogen contents analysed. In a sterile pot experiment, *Bradyrhizobium* symbionts were either inoculated separately or co-inoculated pairwise with PGPR strains *Azospirillum brasiliense* Cd, *Rhizobium laguerreae* PEPV16, *Bacillus amyloliquefaciens* PW1, and *Agrobacterium pusense* IRBG74, or with a consortia. Uninoculated plants and the PGPR consortia without *Bradyrhizobium* served as controls. *Bradyrhizobium* + *R. laguerreae* PEPV16 and the *Bradyrhizobium* + PGPR consortia significantly increased soybean nitrogen accumulation by 24.7% and 24.3%, respectively, compared to the *Bradyrhizobium* alone treatment. There were no detrimental effects of any of the PGPR co-inoculants on biomass traits, although there were significant differences between inoculation treatments and a trend towards improved plant traits in the *Bradyrhizobium* + *R. laguerreae* PEPV16, *Bradyrhizobium* + *A. brasiliense* Cd, and *Bradyrhizobium* + PGPR consortia treatments. This work increases our understanding of

soybean plant-microbial interactions and provides a basis for further tailoring of potential soybean inoculant products.

4.2 Introduction

The agricultural inoculant industry is predicted to increase in value by 0.6 billion USD from 2022 to 2027 (MarketsandMarkets, 2023). A contributing factor to this growth includes increasing concerns over the detrimental effects of the overuse of chemical fertilisers and pesticides on the environment (Gu *et al*., 2023). Applying microorganisms that promote plant growth to agricultural systems can provide crops with essential nutrients (Sammauria *et al*., 2020). Inoculating legumes with symbiotic N_2 fixing bacteria, collectively known as rhizobia, has been an agricultural practice for over 100 years (Herridge, 2008; Kaminsky *et al*., 2019; Santos *et al*., 2019). Soybean is one of the most inoculated crops worldwide, with Brazil leading production of soybean rhizobial inoculants with approximately 36.5 million hectares inoculated annually (Santos *et al*., 2019). Rigorous rhizobia strain isolation programmes, matching strains with crop genotypes and improved legislation surrounding inoculant products has resulted in elite inoculant strains suitable to the local soil conditions and cultivars (Alves *et al*., 2003; Hungria and Mendes, 2015; Siqueira *et al*., 2014). However, soybean growth and inoculation is in its infancy in Northern Europe and particularly the UK, with heavy reliance on the same elite inoculant strains that have been selected for biological nitrogen fixation (BNF) efficiency in South America. Soybean cultivation is on a relatively small scale in the UK, with roughly 8000 acres grown in 2019 confined to warmer regions below the Vale of York (Soya UK, 2019), but there is potential for increased production due to novel early harvesting soybean varieties emerging and a warming climate expanding the range where varieties can be grown (Coleman *et al*., 2021). Therefore, improving inoculant efficiency and increasing the success of the introduced inoculant strains with early harvesting varieties is a priority for the improved use of inoculant technology and soybean production in the UK.

Inoculating legumes with beneficial plant growth promoting rhizobacteria (PGPR) alongside a compatible rhizobia symbiont can boost plant growth (Bai *et al*., 2003; Barbosa *et al*., 2021; Zeffa *et al*., 2020). The aim of co-inoculation is to enhance the BNF capacity of the symbiosis, either by increasing inoculant competitiveness in the rhizosphere, or by providing indirect benefits to the plant, such as essential nutrient acquisition or growth stimulation by phytohormone production. There are several PGPR that have been found to have a positive impact on soybean yield. For example, co-inoculation of *Bradyrhizobium japonicum* with *Bacillus amyloliquefaciens* LL2012 increased soybean nodule numbers synergistically, with B. *amyloliquefaciens* LL2012 found to produce significant levels of phytohormones involved in plant growth and defence (Masciarelli *et al*., 2014; Sabaté *et al*., 2017; Tiwari *et al*., 2017). Similarly, other *Bacillus* species have been found to enhance nodulation and plant yields in field conditions (Bai *et al*., 2003). *Azospirillum brasilense* is a free-living diazotroph that is already widely employed as a co-inoculant with *Bradyrhizobium* species in Brazil (Barbosa *et al*., 2021; Hungria *et al*., 2013; Moretti *et al*., 2020; Santos, 2021). *Azospirillum brasilense* can produce and secrete phytohormones, such as auxins, into the rhizosphere, which can stimulate plant root growth (Fukami *et al*., 2018; Santos, 2021). Increasing root surface area can also enhance the opportunity and interaction with nodulating bacteria (Chibeba *et al*., 2015; Rondina *et al*., 2020; Santos, 2021). When *A. brasilense* strains Ab-V5 and Ab-V6 were co-inoculated with *Bradyrhizobium sp.* significant changes in root morphology resulted in significantly higher nodule numbers (Rondina *et al*., 2020). Other rhizobia species have been found to possess PGPR qualities when inoculated onto a range of crops, not just their legume host species. For example, *Rhizobium laguerre* strain PEP16V originally isolated from *Phaseolus vulgaris* has been found to improve vitamin contents in strawberry (Flores-Félix *et al*., 2018) and increase lettuce and carrot plant biomass (Flores-Félix *et al*., 2021). Additionally *Agrobacterium (syn. Rhizobium) pusense* strain IRBG74, a rhizobial symbiont of the aquatic legume *Sesbania* sp. (Cummings *et al*., 2009) has been found to promote rice (Biswas *et al*., 2000; Crook *et al*., 2013; Mitra *et al*.,

2016) and mung bean growth (Chaudhary *et al*., 2021). These results suggest that inoculating a combined, diverse consortia of PGPRs with compatible rhizobia may result in greater plant yields than rhizobial inoculation alone.

In recent years the concept of inoculating crops with a diverse bacterial consortia has become more popular than single species/ strain inoculations. The soil microbial community is one of the most diverse microbiomes on earth and a decline in soil bacterial diversity has been found to decrease plant productivity (Chen *et al*., 2020). Introducing a consortia of diverse bacteria with complementary functions can enhance multiple beneficial functions simultaneously in the microbiome, for example improving BNF, phosphate solubilisation, siderophore production, and mitigation of biotic and abiotic stressors (Xavier *et al*., 2023). Alternatively, inoculating diverse consortia with redundant functions can also increase the chances that at least one of the strains capable of key functions will survive in the complex soil microbiome, and hence avoid competitive exclusion (Fields *et al*., 2021; Hu *et al*., 2016; Kaminsky *et al*., 2019). Although it is extremely hard to predict how diverse inocula would function across a range of environments, for legume inoculant production it is crucial at the inoculant development stage to assess whether additional PGPR strains are compatible with the rhizobial symbiont and do not antagonise the symbiosis. Here, a range of PGPR and their impact on soybean plant growth traits when co-inoculated with *Bradyrhizobium* species was assessed in a greenhouse pot experiment. Hypotheses include; that no detrimental effects of co-inoculation on soybean plant biomass traits will be observed and potentially an improvement in soybean plant biomass traits, particularly in the *Bradyrhizobium* + *Azospirillum* co-inoculation treatment is expected.

4.3 Methods

Rhizobia and PGPR strains

Two *Bradyrhizobium* species were used in combination: *Bradyrhizobium diazoefficiens* (GW50) and *Bradyrhizobium japonicum* (GW140). These strains are natural variants of *B. diazoefficiens* SEMIA 5080 and *B. japonicum* SEMIA 5079 that were isolated from UK soils following a soybean cropping season (isolation described in Chapter 2). A dual *Bradyrhizobium* inoculation strategy is currently a widespread inoculation strategy for soybean in the UK (Legume Technology Ltd.). PGPR strains included; *Azospirillum brasiliense* Cd*, Rhizobium laguerreae* PEPV16, *Bacillus amyloliquefaciens* PW1 and *Agrobacterium* (previously *Rhizobium*) *pusense* IRBG74 due to their previously ascribed PGPR qualities (Table 1) and were supplied by PlantWorks UK. *Bradyrhizobium* strains were streaked out from glycerol stocks and incubated for 5 days on yeast mannitol (YM) agar plates (Table S1) at 28°C, single colonies were inoculated in 100 mL of YM broth and grown in a shaker-incubator 180 rpm at 28°C for 5 days. PGPR strains were cultured on nitrogen-free agar (NFa) plates (Table S1) and single colonies inoculated into 100 mL tryptone yeast broth in a shaker-incubator 180 rpm at 28°C for 3 days. Strains were standardised to 10^8 colony forming units (CFU) mL^{-1} and resuspended in phosphate buffer saline (PBS) for inoculation.

Table 1. PGPR qualities of inoculant species used in this study.

PGPR species	PGPR qualities	Plant host studied	References
Azospirillum brasilense	•Diazotrophic •Indole Acetic Acid + other phytohormone production •Phosphorus solubilsation •Siderophore production •Induction of antioxidant plant enzymes	• *Glycine max L. merr* (Soybean) • *Triticum aestivum* (Wheat) • *Phaseolus vulgaris* (Common bean) • Broad host plant range - increased growth in 113 plant species spanning 35 botanical families	Turan *et al.*, 2012; Hungria, Nogueira and Araujo, 2013; Pereg, de-Bashan and Bashan, 2016; Fukami, Cerezini and Hungria, 2018
Rhizobium languerre	•Siderophore production •Indole Acetic Acid production •Increased micronutrient elements and organic acids •Symbiotic N^2 fixation	• Lactuca sativa L. (Lettuce) • Daucus carota L. (Carrot) • Fragraria x ananassa (Strawberry) • *Phaseolus vulgaris* (Common bean) • *Lens culinaris* (Lentil)	Flores-Félix *et al.*, 2013, Flores-Félix *et al.*, 2018, Flores-Félix *et al.*, 2021, Taha *et al.*, 2022
Agrobacterium (syn. Rhizobium) pusense	•Symbiotic N^2 fixation •Indole Acetic Acid production •Phosphorus solubilsation •Siderophore production •Ammonia production •ACC deaminase production	• *Sesbania cannabina* • *Vigna radiata* (Mung bean) • *Orzya sativa* (Rice)	Cummings et al., 2009, Mitra et al., 2016, Chaudhary et al., 2021
Bacillus amyloliquefaciens	•Indole Acetic Acid + other phytohormone production •ACC deaminase production •Siderophore production	• *Glycine max L. merr* (Soybean) • *Orzya sativa* (Rice) • *Phaseolus vulgaris* (Common bean)	Masciarelli et al 2014 Tiwari et al., 2017 Sabaté et al., 2017

Experiment design and set-up

Eight treatments were established to assess beneficial combinations of PGPRs with *Bradyrhizobium* strains. Treatments included: no bacteria controls (sterile PBS), *Bradyrhizobium* inoculation (GW50 + GW140), *Bradyrhizobium* + *Agrobacterium* IRBG74, *Bradyrhizobium* + *Rhizobium* PEPV16, *Bradyrhizobium* + *Azospirillum brasilense*, *Bradyrhizobium* + *Bacillus amyloliquefaciens, Bradyrhizobium* + consortia of PGPR and the PGPR consortia without *Bradyrhizobium*. The uninoculated controls had 20 replicates whilst the bacteria treatments each had 10 plant replicates, resulting in a total of 90 pots. Soybean seeds of the early harvesting variety Siverka, currently the most widely grown soybean variety in the UK, were provided by Soya UK. Seeds were sterilised by shaking in 70% EtoH for 1 minute followed by 25 min shaking in 10% household bleach, washed 6 times with sterile dH_2O and germinated on 0.5% agar plates for 3 days at 25°C. Seedlings were planted into a twice-autoclaved mixture of sand and vermiculite (4:1) and inoculated with 1 mL of inoculant, which was prepared with equal volume mixtures of each strain (10^8 CFU mL^{-1}). The experiment was split into two blocks with half the replicates planted on day 1 and the other half planted on day 4, each block was harvested at 8 weeks. Plants were grown in sterilised clear polythene bags to prevent cross contamination and supplied weekly with 20 mL of a N-free nutrient solution CRS (Howieson and Dilworth, 2016), until week 5, and 40 mL thereafter. At harvest, aboveground biomass was separated from below-ground biomass. Pods were counted and removed from aboveground parts; both were dried at 80°C for 48 hours. When dried, seeds were removed from pods and weighed separately. Nodules were counted, removed from plant roots, and dried separately to root biomass at 80°C for 48 hours. For two replicates in the consortia treatment, 5 root nodules were sampled before drying and isolates extracted (see below). Aboveground biomass was pooled and ground for %N analysis on an isotope ratio mass spectrometer (ANCA GSL 20-20 Mass Spectrometer; Sercon Cheshire).

Isolation and identification of nodule bacteria

During the experiment two out of ten replicates of the consortia without *Bradyrhizobium* had visibly improved growth traits and were found to possess nodules at harvest. Therefore to check for the presence of *Bradyrhizobium* symbionts, nodule bacteria were isolated by sampling 5 root nodules per plant, sterilising (1 min in 70% EtOH, 3 mins 2.5% NaOCl, 6 sterile dH_2O washes) and crushing in 750µL of sterilised 10 mM MgSO4 and 0.01% tween solution (Howieson and Dilworth, 2016). Five µL of crushed nodule mixture and a 10^{-5} dilution was streaked onto yeast mannitol agar plates (Howieson and Dilworth, 2016). Plates were left to grow at 28°C for 5 days, then different colony morphologies were repeatedly streaked until single isolates were obtained. Colony BOXPCR (Versalovic *et al*., 1994) was conducted to identify whether a *Bradyrhizobium* symbiont was present by comparison to the two inoculant reference BOXPCR patterns. DNA products were visualised on a 2% Agarose gel in Tris-borate-EDTA (TBE) buffer stained with SYBRsafe dye, run at 90V for 1 hour and 50 minutes. Colonies were also screened for the presence of soya-specific *Bradyrhizobium nodZ* (BnodZ) to check for horizontal gene transfer of a symbiosis gene. To identify colonies that did not match reference strain BOXPCR patterns, the *16S rRNA* region (Heuer *et al*., 1997) was sequenced (Azenta, UK). Primers and PCR conditions can be found in Table S2, sequences were identified using NCBI blast (Altschul *et al*., 1990).

Data analysis

Aboveground N biomass accumulation (g) was calculated as follows:

(%N / 100) x (shoot biomass + pod biomass).

Values for mg N g^{-1} plant dry weight were calculated as follows:

1. (%N / 100) x mg of sample analysed = mg N dry weight
2. (mg N dry weight / mass of sample analysed) x 1000 = mg N g^{-1} plant dry weight.

Delta ^{15}N values (‰) in this study were calculated using internal standards where absolute isotope ratios are measured for sample and standard (atmospheric air) and the relative measure of delta is calculated thus:

$\delta^{15}N$ (‰) vs [std] = (R_{sample} - R_{std}) / R_{std} where R = (At%^{15}N / At%^{14}N).

All data were analysed on R (v 4.1.3) with R studio (R Studio Team, 2020). Linear models were constructed to test the effect of inoculation treatment and planting block on plant growth responses and assessed using the 'Anova' function from the car package (Fox and Weisberg, 2019), after checking data conformed to the assumptions of the test. 'Anova' in the car package performs a type II test ANOVA to test for all the variance associated with the first dependent variable (treatment) then all the variance associated with the next dependent variable (block) rather than assessing the variance sequentially. Harvest index is expressed as the percentage of biomass invested in seeds and was calculated as seed biomass / total biomass multiplied by 100. Root: shoot ratio was calculated as root biomass / shoot biomass. Contrasts between coefficients in the linear models (*t* and *p*- values) are reported to support specific treatment effects. Replicates that had potential cross-contamination (2/10 in the consortia treatment) were removed from the analysis apart from where explicitly stated. Post-hoc tests using Tukey's Honestly Significant Difference test were conducted to find significant differences between the treatments using the agricolae package (Felipe de Mendiburu and Muhammad Yaseen, 2020). Graphs were produced using ggplot2 (Wickham, 2016). A linear mixed effects model (LMM) was constructed using the package lme4 (Bates *et al*., 2015), to assess the effect of N biomass and inoculation treatment on seed biomass, whilst accounting for planting block as a random effect. 'Anova' in the car package was used for treatment effect sizes and *p*-value generation of the LMM (Fox and Weisberg, 2019).

4.4 Results

Inoculation impacts on plant biomass

All *Bradyrhizobium* + PGPR treatments yielded statistically similar plant biomass traits to the *Bradyrhizobium* alone treatment, highlighting that there was no direct detrimental effect of using diverse inocula on soybean biomass traits in this controlled experiment (Figure 1). The uninoculated control and consortia only consistently yielded lower plant biomass traits (Figure 1). The exception to this was root biomass (Figure 1), where there was a significant effect of treatment (ANOVA, $F_7 = 14.26$, $p < 0.001$) and the control and consortia treatments had significantly larger root biomass than all the treatments where *Bradyrhizobium* was included ($t = 9.2$, $p < 0.001$). There was a significant effect of treatment on shoot (ANOVA, $F_7 = 19.91$, $p < 0.001$) and total plant biomass (ANOVA, $F_7 = 45.31$, $p < 0.001$), where the *Bradyrhizobium* + PGPR consortia treatment yielded the overall largest shoot and total biomass, which was not significantly different from the *B* + *Azospirillum*, *B* + *Rhizobium* and *Bradyrhizobium* alone treatments (Figure 1). There was also a significant effect of inoculation treatment on seed biomass (ANOVA, $F_7 = 71.5$, $p < 0.001$), where the *B* + *Rhizobium* treatment yielded the largest seed biomass, which was significantly larger than the *B* + *Bacillus* treatment, but not significantly different from the *Bradyrhizobium* alone, *B* + *Azospirillum, B* + *Agrobacterium* and *B* + consortia treatments (Figure 1). Harvest index also indicated that all inoculation treatments containing *Bradyrhizobium* invested significantly more biomass into seed production (Figure S1). There was a significant effect of planting block on all biomass traits except seed biomass, with pots planted in block 2 having higher plant biomass traits overall, which did not mask treatment effects. A different batch of sand was used to make up the block 2 pots, which may have varied in micronutrient elements.

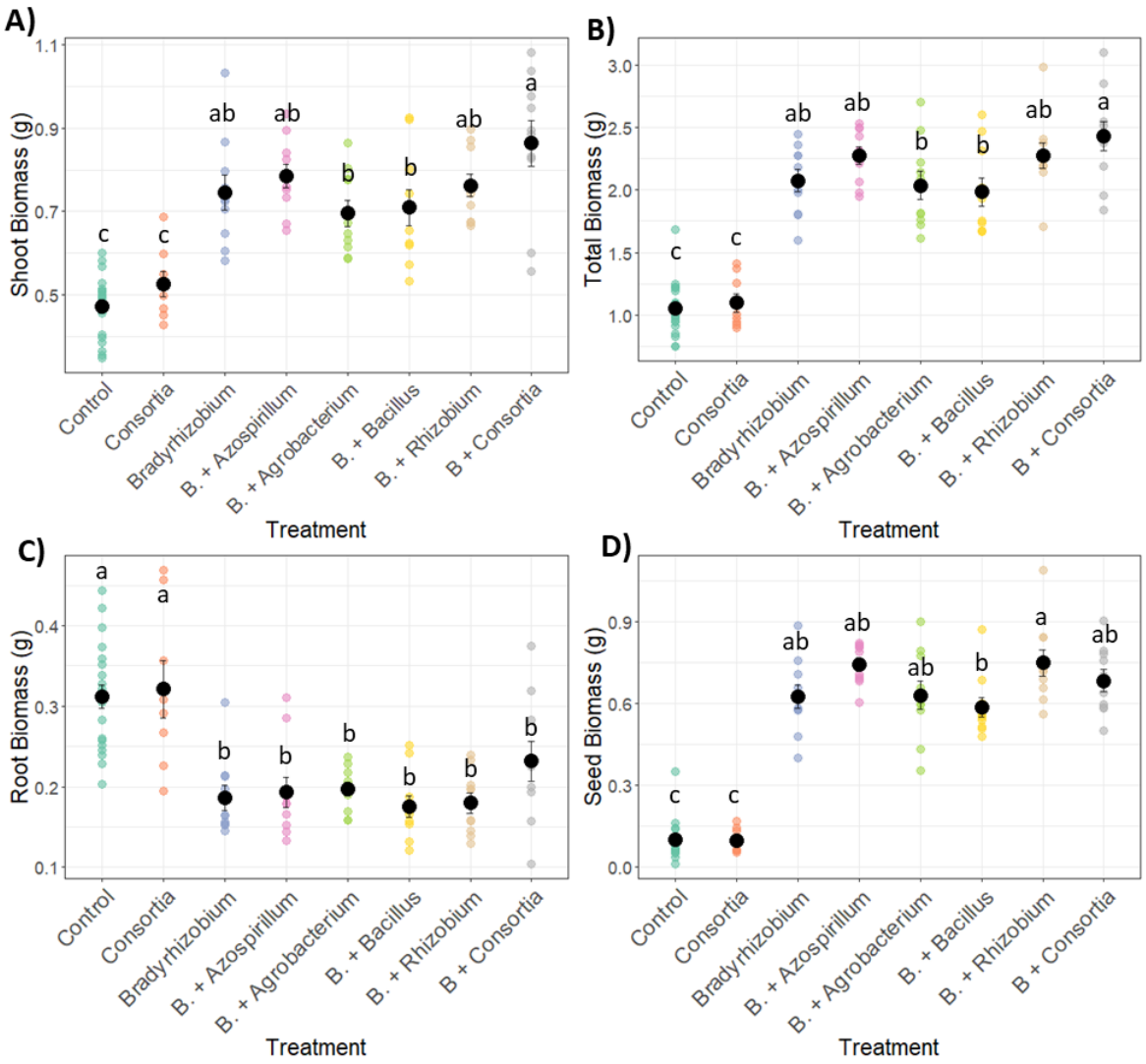

Figure 1. Comparison of inoculation treatments on soybean ***A)*** shoot, ***B)*** total, ***C)*** root and ***D)*** seed biomass. Different letters denote Tukey post-hoc significance ($p < 0.05$) tests.

Inoculation impacts on symbiotic traits

No root nodules were present on control plants, however two out of ten replicates in the consortia alone treatment possessed nodules, these were removed from the analysis. PGPR inoculation treatment had no significant effect on nodule biomass and nodule numbers (Figure 2A). There is a trend towards higher nodule biomass in the *B* + consortia treatment compared to the *Bradyrhizobium* alone treatment, however there is a large amount of variation within this treatment. There was a significant positive relationship between

nodule biomass and total plant biomass (y = 0.80 + 22.53x, $F_{1, 58}$ = 131.69, p < 0.0001) and also between nodule biomass and seed biomass (y = 0.33 + 5.45x, $F_{1, 58}$ = 19.3, p < 0.0001), although there was no significant influence of inoculation treatment (Figure 2B).

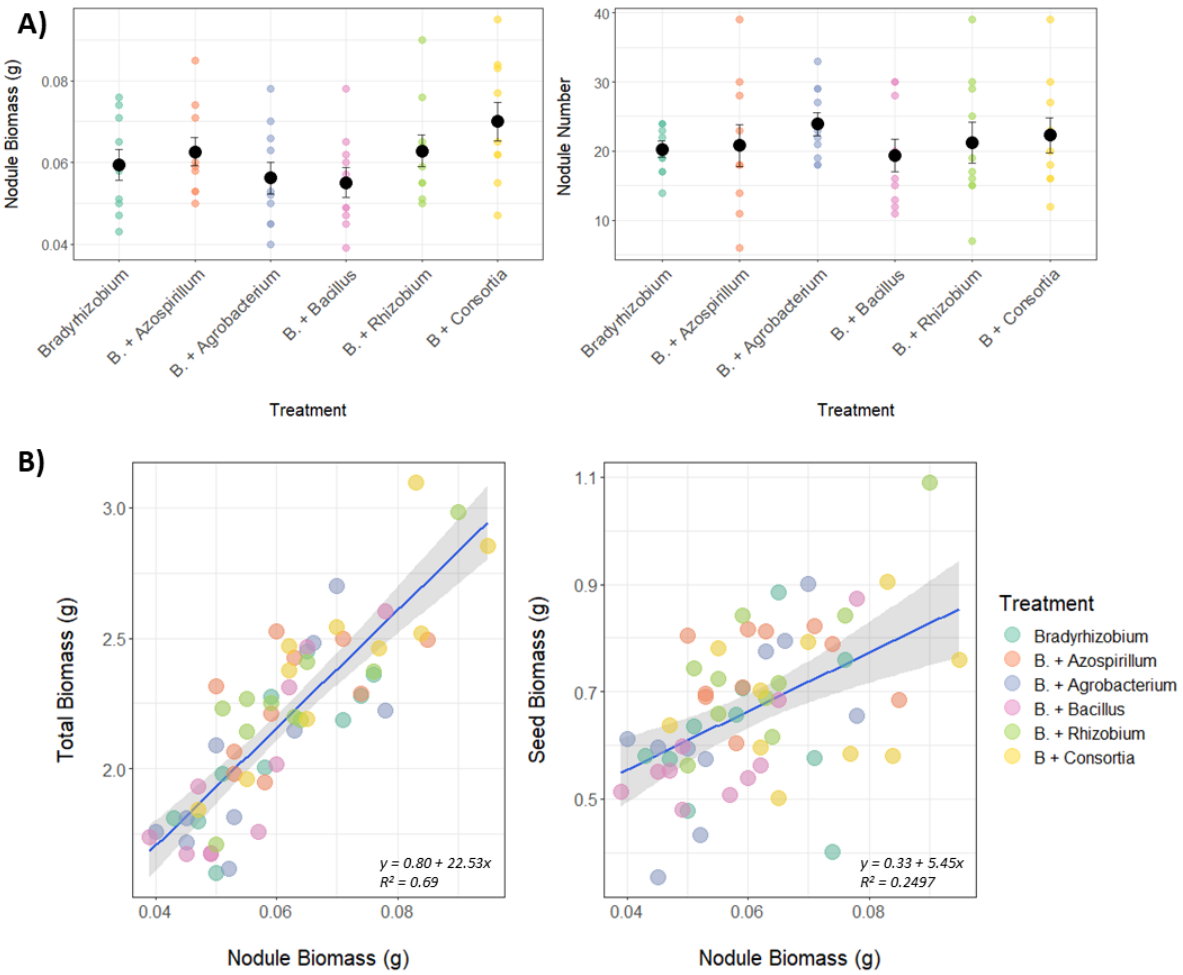

Figure 2. A) Comparison of inoculation treatments on symbiotic traits, nodule biomass and nodule number. There were no significant differences between inoculation treatments. ***B)*** Significant positive relationships between nodule biomass and total plant biomass and nodule biomass and seed biomass.

Inoculation impacts on nitrogen content

The aboveground nitrogen content of PGPR inoculated plants differed, with the *B* + consortia and *B* + *Rhizobium* treatment yielding the largest aboveground N biomass accumulation, which was significantly increased from the *Bradyrhizobium* alone and B +

Agrobacterium treatment (see Figure 3A and Table 2). When standardised by plant weight and expressed as mg N/ g^{-1} plant biomass, none of the PGPR treatments had statistically significant differences compared to the *Bradyrhizobium* alone treatment, but B + *Bacillus* and B + *Rhizobium* were significantly increased from the B + *Agrobacterium* treatment (Figure 3B). All treatments containing *Bradyrhizobium* had significantly higher N biomass and mg N/ g^{-1} plant biomass than those without (Table 2, Figure 3).

Measurements of the stable N isotopes within the plant tissue allowed quantification of δ^{15}N (‰). As the inoculated plants in this experiment have been grown in a system where most plant N (apart from seed and residual N) is derived from symbiotic N_2 fixation, we expect a similar δ^{15}N value to its N source, the atmosphere (0‰) (Unkovich *et al*., 2008). All treatments inoculated with N_2 fixing *Bradyrhizobium* had significantly lower δ^{15}N (‰) values than the control and consortia treatments, ranging from -0.68 in the *Bradyrhizobium* treatment to -0.92 in the B + consortia treatment (Figure 3C and Table 2). There is increased mg N/ g^{-1} plant with lower δ^{15}N values (Figure 3D), highlighting how plants that acquired N from the atmosphere yielded larger mg N/ g^{-1} plant weight.

There are some emerging trends from the panel of PGPR assessed in this study which could help fine-tune inoculum formulas going forwards. For example, when assessing the influence of aboveground N accumulation and inoculation treatment on an agronomically important trait like seed biomass, the *B + consortia*, *B + Rhizobium* and *B + Azospirillum* treatments yielded the largest N accumulation and seed biomass compared to the other treatments (Figure 4). When assessed using a linear mixed effects model with planting block as a random effect, there was a significant effect of N accumulation (ANOVA type II; χ^2 = 66.6, *d.f.* = 1, $p < 0.0001$), and inoculation treatment (ANOVA type II; χ^2 = 30.4, *d.f.* = 7, $p < 0.0001$) on seed biomass, with no significant interaction.

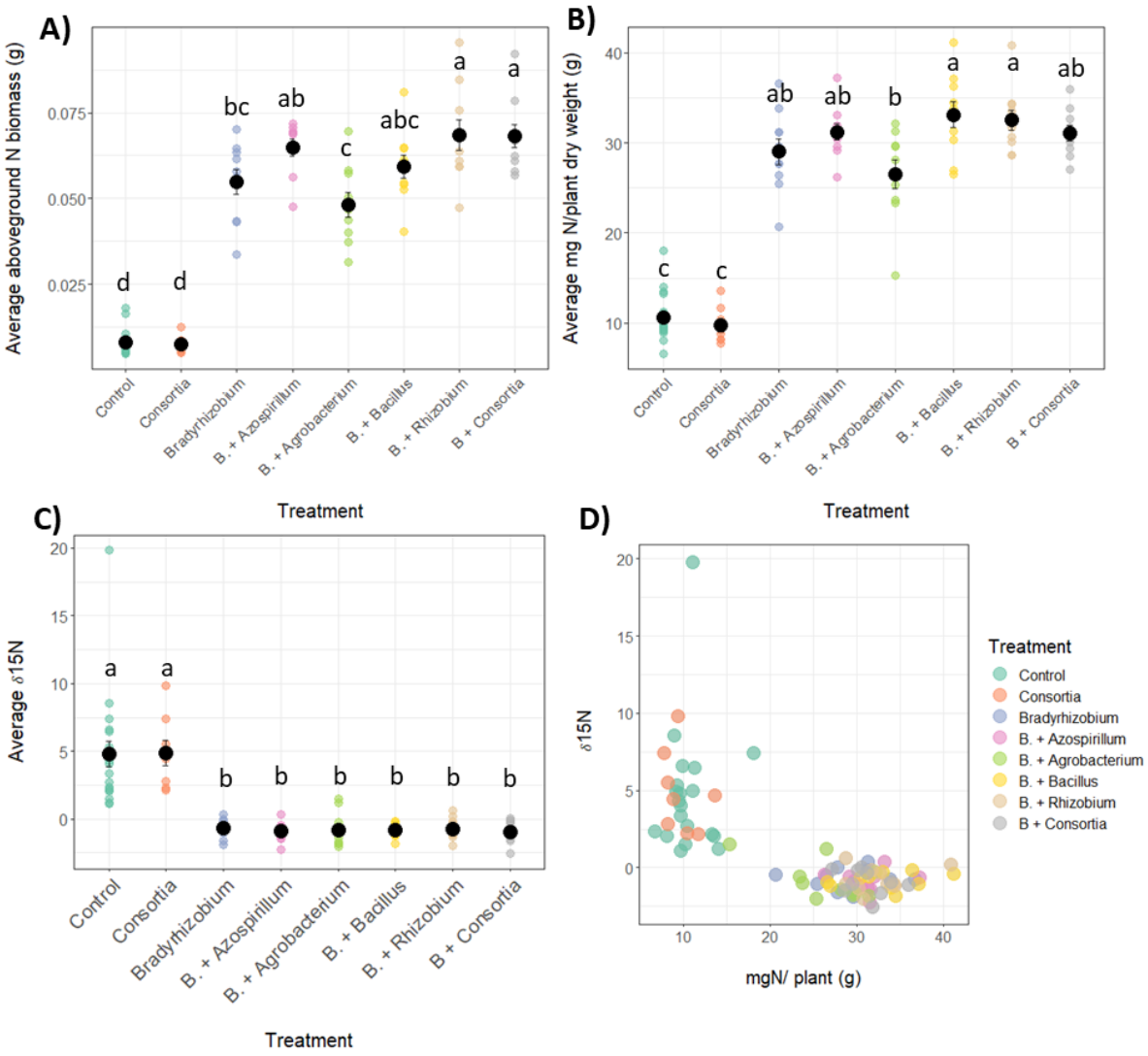

Figure 3. Plant N traits under different inoculation treatments. **A)** Average aboveground nitrogen plant biomass, **B)** Average mg N/ g^{-1} plant dry weight, **C)** Average $\delta^{15}N$ (‰) under different inoculation treatments. Black dots represent means, error bars represent standard errors and coloured dots represent individual replicates within treatments. Different letters denote Tukey post-hoc significance ($p < 0.05$) tests. **D)** Scatterplot of mg N/ plant dry weight and $\delta^{15}N$ (‰) values coloured by different inoculation treatments.

Table 2. Soybean nitrogen traits under different inoculation treatments. Averages for controls (n = 20) and inoculation treatments (n = 10), ± standard errors. Different letters denote Tukey post-hoc significance ($p< 0.05$) tests. ANOVA type two statistics for each trait listed below.

	Average Soybean (Siverka) N contents		
Treatment	**Aboveground N accumulation (g)**	**mg N / g^{-1} plant dry weight**	**δ^{15}N (‰)**
Control	0.008076 ± 0.0008 *d*	10.6125 ± 0.56 *c*	4.78635 ± 0.92 *a*
Consortia	0.007645 ± 0.0008 *d*	9.7375 ± 0.72 *c*	4.884875 ± 0.94 *a*
Bradyrhizobium (GW140 / SEMIA 5079 + GW50 / SEMIA 5080)	0.054874 ± 0.0036 *bc*	29.017 ± 1.43 *ab*	-0.676 ± 0.22 *b*
B + *Azospirillum*	0.064845 ± 0.0024 *ab*	31.177 ± 0.90 *ab*	-0.8938 ± 0.22 *b*
B + *Agrobacterium*	0.04822 ± 0.0035 *c*	26.482 ± 1.57 *b*	-0.7818 ± 0.40 *b*
B + *Bacillus*	0.059378 ± 0.0033 *abc*	33.084 ± 1.43 *a*	-0.8321 ± 0.16 *b*
B + *Rhizobium*	0.068424 ± 0.0044 *a*	32.515 ± 1.13 *a*	-0.7374 ± 0.25 *b*
B + Consortia	0.068183 ± 0.0033 *a*	31.08 ± 0.82 *ab*	-0.9219 ± 0.26 *b*
ANOVA type II tests			
Treatment (*d.f.* = 7)	F = 102.4 p < 0.0001	F = 94.5 p < 0.0001	F = 16.7 p < 0.0001
Block (*d.f.* = 1)	F = 7.84 p < 0.01	F = 1.72 p = 0.2	F = 3.02 p = 0.08

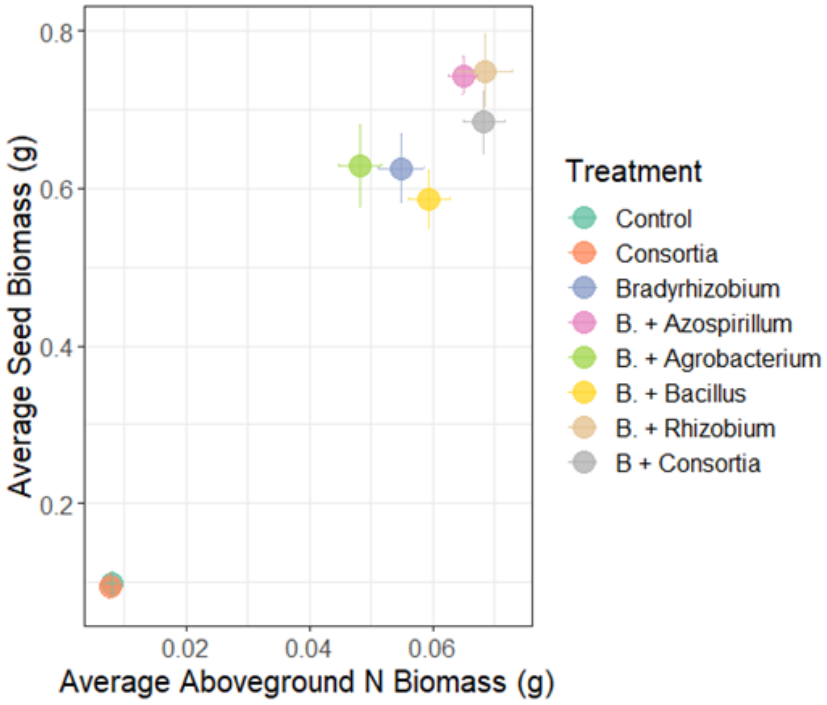

Figure 4. Average aboveground N biomass versus average seed biomass. Colour = Inoculation treatments. Errorbars are standard errors.

Evidence for co-infection

During the experiment two out of ten replicates treated with PGPR consortia without *Bradyrhizobium* treatment turned green after 6 weeks, while the other plants in this treatment were visibly nitrogen deficient (*i.e.* yellow); these two replicates were found to have root nodules at harvest. Five nodules from each plant were harvested, sterilised, and crushed to extract the nodule bacteria to assess whether *Bradyrhizobium* species were present. The highly competitive *Bradyrhizobium japonicum* (GW140) strain was present in the nodules of both plants, suggesting this symbiont was responsible for the nodule formation (Figure S2). Two non- *Bradyrhizobium* colonies were isolated from the nodule extracts (highlighted in red and blue in Figure S3A). Sequencing of the *16S rRNA* gene region followed by BLAST of the resulting sequences putatively identified a *Microbacterium hydrocarbonoxydans* species (found only in replicate 9; blue) and *Rhizobium pusense* IRBG74 (*Agrobacterium*) isolate

which was found in the nodules of both replicates (red; Table S3). The *Bradyrhizobium nodZ* PCR proved positive only for the *B. japonicum* isolates (Figure 3B).

4.5 Discussion

Here, a panel of PGPR and their effect as co-inoculants with soya-nodulating *Bradyrhizobium* strains on a temperate-adapted soybean cultivar was investigated. As expected, the uninoculated controls and the PGPR consortia without *Bradyrhizobium* yielded the lowest plant biomass and N contents. With the exception of two plants, the control and consortia plants appeared nitrogen deficient (yellow), and the increased root biomass, root: shoot ratio and low harvest index highlights how these plants invested more in the roots to try and access more nutrients. In contrast, the supply of fixed N through the nodules of the *Bradyrhizobium*-inoculated treatments meant they could invest around 30% of their fixed carbon biomass to seed production (refer to Figure S1). None of the tested PGPR significantly decreased yields or plant N contents compared to the standard *Bradyrhizobium* alone treatment, suggesting the potential for all to be used as co-inoculants. However, for aboveground N biomass, the B + *Rhizobium* and B + consortia treatments gave significantly higher aboveground biomass N accumulation than the *Bradyrhizobium* alone treatment, with average increases of 24.7% and 24.3%, respectively. When standardising for plant weight using mg N/ g^{-1} dry weight, significant differences are no longer observed, suggesting that the higher aboveground biomass in the B + *Rhizobium* and B + consortia treatments is driving this effect, rather than N content. Additionally, there is a trend towards higher seed biomass and aboveground N biomass accumulation in the B + *Azospirillum*, B + *Rhizobium* and B + consortia treatments.

This study provides the first evidence of a beneficial association between *Rhizobium laguerreae* strain PEPV16 (previously *R. leguminosarum*) and *Bradyrhizobium* species for soybean inoculation. *R. laguerreae* PEPV16 has been shown to be a beneficial PGPR strain

for other non-legume crops such as strawberry (Flores-Félix *et al*., 2018), carrot and lettuce (Flores-Félix *et al*., 2021), in addition to its role as a rhizobial symbiont for common bean and lentils (Flores-Félix *et al*., 2019; Taha *et al*., 2022). Plants in the B + *Rhizobium* treatment had significantly larger aboveground N biomass accumulation than the *Bradyrhizobium* alone treatment, suggesting synergistic increases in plant performance with co-inoculation. Additionally, B + *Rhizobium* plants also had a trend towards larger biomass traits, including the largest seed biomass which was a significant increase of 27.7% compared to the *B* + *Bacillus* treatment. PEPV16 has been shown to harbour multiple genes associated with phosphorus solubilisation and many other genes involved in plant colonisation (Flores-Félix *et al*., 2021), and has been found to increase certain organic acids in strawberries (Flores-Félix *et al*., 2015). However, the mode of action of its PGPR capabilities with *Bradyrhizobium* species on soybean are yet to be determined. Similarly, the B + consortia treatment yielded significantly larger aboveground N accumulation compared to the *Bradyrhizobium* alone treatment and trended towards larger biomass traits. This could be the result of the beneficial *Bradyrhizobium*- *R. laguerreae* interaction in the consortia, for example diverse rhizobia inoculum performance can be predicted by the best performing individuals within a consortia (Fields *et al*., 2021). However, it could also be due to subtle, additive effects of PGPR in the consortia. These results look promising for testing the capacity of the diverse consortia under field conditions, where there may be more benefits in a complex environment.

No significant improvements in plant biomass or nitrogen content were observed when *Bradyrhizobium* + *Azospirillum* strains were co-inoculated in comparison to the *Bradyrhizobium* alone treatment, but there were trends towards improved traits (refer to Figure 4). Previous research suggests co-inoculation of *Azospirillum* and *Bradyrhizobium* species significantly improves soybean yields, and their co-inoculation is currently a widespread strategy used for soybean inoculation, particularly in South America (Chibeba *et al*., 2015; Hungria *et al*., 2013; Santos, 2021). A meta- analysis revealed that co-inoculation

of *Bradyrhizobium* with *Azospirillum* in Brazilian field conditions primarily increases root growth and nodulation, which are the main factors leading to increased soybean grain N content and yields (Barbosa *et al*., 2021). Additionally, higher efficiency of co-inoculation is observed when *Azospirillum* is co-inoculated with *B. japonicum* and *B. diazoefficiens* species, rather than with *B. japonicum* and *B. elkanii* or just *B. japonicum* alone (Barbosa *et al*., 2021), which might be linked to the higher N_2 fixation capacity of the elite *B. diazoefficiens* inoculant strains (Siqueira *et al*., 2014). *Azospirillum* species are broad-acting PGPR which are beneficial for a range of agronomically relevant crops (Pereg *et al*., 2016). They have been reported to possess multiple PGPR traits including free living N_2 fixation, phosphorus solubilisation (Turan *et al*., 2012), phytohormone production (Perrig *et al*., 2007) and bio-control of plant pathogens (Pérez-Montaño *et al*., 2014). However, as with many PGPR interactions these can vary with bacteria strain, plant cultivar, soil environment, and climatic conditions (Fukami *et al*., 2018). The benefits of co-inoculation with *Azospirillum* may improve in more complex conditions where there is a diversity of nutrients and interacting microbial species. For example, inoculation of maize (Zea mays L.) with *A. lipoferum CRT1* increased the diazotrophic community size in soils which enhanced plant yields but was site-specific (Renoud *et al*., 2022). Therefore, plants may receive indirect benefits from inoculant strains co-assembling beneficial microbiomes, thus experiencing an accumulation of multiple beneficial traits (Cassán *et al*., 2020). Considerably less research has investigated the effect of *Bradyrhizobium* and *Azospirillum* co-inoculation of soybean in temperate regions. In a midwestern US study assessing 25 field trials, no field showed a significant difference between *Bradyrhizobium* inoculation alone and co-inoculation with *Azospirillum* (de Borja Reis *et al*., 2022). More research into *Bradyrhizobium* and *Azospirillum* co-inoculation for soybean in temperate regions is needed, particularly where their inoculation and introduction is relatively new, like the UK, as the beneficial traits observed in the tropics may not be replicated in different climatic conditions.

An interesting result was the occurrence of the *Agrobacterium pusense* strain in the nodules of two plant replicates of the consortia treatment that had become cross-contaminated with *Bradyrhizobium* symbionts. As *A. pusense* was present in the consortia inoculant, this may highlight the potential for this strain to co-infect and populate nodules when a compatible soybean rhizobium is present, as other rhizobia strains have been found to do (Gano-Cohen *et al*., 2016; Zgadzaj *et al*., 2015). On the other hand the *Microbacterium* strain that was isolated from only one plant replicate may be an opportunistic nodule coloniser (Mayhood and Mirza, 2021) that survived the autoclaving of growth material, or it was introduced under the non-sterile greenhouse conditions.

4.6 Conclusion

This study assessed a panel of known PGPR strains in combination with *Bradyrhizobium* inoculants for a temperate adapted soybean cultivar. Across the PGPR panel, consisting of *Azospirillum brasiliense* Cd*, Rhizobium laguerreae* PEPV16, *Bacillus amyloliquefaciens* PW1 and *Agrobacterium pusense* IRBG74, none significantly decreased plant biomass traits or plant N accumulation when compared to *Bradyrhizobium* inoculation alone. Additionally, co- inoculation of *Bradyrhizobium* spp. with *R. laguerreae* PEPV16 and the whole consortia yielded the largest aboveground nitrogen biomass accumulation, suggesting beneficial PGPR attributes in this pot experiment. Future work should focus on assessing this diverse consortia in an ecologically relevant context, such as in live soil or field conditions. The δ^{15}N values produced in this study could be used for 'B' values for the early harvesting soybean cultivar Siverka, as these were grown in N- free conditions and inoculated with highly similar variants of SEMIA 5080 and SEMIA 5079, one of the most widely employed strategies across the UK. This would be valuable for quantifying how much N in field-grown soybeans is derived from the atmosphere and thus the symbiosis (Unkovich *et al*., 2008). Research presented here increases our understanding of rhizobia- PGPR interactions and may be useful for the formulation of soybean inoculant products.

4.7 Acknowledgements

I would like to thank Plantworks UK for collaborating on this experiment and providing the PGPR strains. I would like to thank Amy Leckie for help setting up a trial experiment and Dr Q Lin for her advice and direction with the project.

4.8 Supplementary Information

Table S1. Growth media for inoculant strains

Media	Formula	Bacterial Strain
Yeast Mannitol Agar/ Broth	Mannitol - 5g/L K_2HPO_4 - 0.5 g/L $MgSO_47H_2O$ - 0.2 g/L NaCl - 0.1 g/L Yeast - 0.4 g/L Agar - 12 g/L (omitted for broth) Distilled water to 1L	*Bradyrhizobium japonicum* GW140 *Bradyrhizobium diazoefficiens* GW50
NFa	K_2HPO_4 - 0.2 g/L KH_2PO_4 - 0.6 g/L $MgSO_47H_2O$ - 0.2 g/L $FeCl_3$ - 0.01 g/L $Na_2Mo_42H_2O$ - 0.02 g/L Yeast - 0.05 g/L $CaCl_2$ - 0.02 g/L Malic acid - 5 g/L Sucrose - 5 g/L Vitamin - 1mL/L •To 100ml add 0.01g of biotin and 0.02g of Pyridoxal-HCl Agar - 13g/L pH 6.5 Distilled water to 1L	*Azospirillum brasiliense* Cd *Rhizobium laguerreae* PEPV16 *Bacillus amyloliquefaciens* PW1 *Agrobacterium pusense* IRBG74
Tryptone -Yeast Agar/Broth	Tryptone - 5 g/L Yeast - 3g/L NaCl - 5 g/L Agar - 12 g/L (omitted for broth) Distilled water to 1L	*Azospirillum brasiliense* Cd *Rhizobium laguerreae* PEPV16 *Bacillus amyloliquefaciens* PW1 *Agrobacterium pusense* IRBG74

Table S2. Primers and PCR conditions used in this study

Primer Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	PCR reaction	PCR programme	Reference
BOXAIR	CTACGGCAAGGCGACGCT GAC G	N/A	• 15 µL GoTaq master mix • 2.4 µL 10 mM primer • 10.6 µL PCR-grade water • 2 µL boil prepared single colony	94°C for 30s 35 x (94°C for 10s, 50°C for 30s, 72°C for 30s) 72°C for 10min	Versalovic et al., 1994
27f + 1492r (16S)	AGAGTTTGATCCTGGCTC AG	TACGGYTACCTTGTTACG ACTT	• 12.5 µL GoTaq master mix • 1 µL F 10 mM primer • 1 µL R 10 mM primer • 8.5 µL PCR-grade water • 2 µL boil prepared single colony	94°C for 5 mins 30 x (94°C for 10s, 55°C for 30s, 72°C for 30s) 72°C for 10 mins	Heuer et al., 1997
BnodZ2	TCGTCCTCGAGCAGGTTT CGGTTAA	CGAAGCCATAAGCGCTT GCGAGT	• 12.5 µL GoTaq master mix • 1 µL F 10 mM primer • 1 µL R 10 mM primer • 8.5 µL PCR-grade water • 2 µL boil prepared single colony	95°C for 5 mins 30 x (95°C for 10s, 69°C for 30s, 72°C for 30s) 72°C for 5 mins	Chapter 1

Table S3. 16S sequences identified from isolates extracted from the consortium minus *Bradyrhizobium* replicates 9 and 10.

Isolate	Sequence (FASTA)	NCBI Top 5 hits (megablast, 28/03/22)
C-B9(3)	>C-B 9 (3)| 1132 bp TCNNAACGGTGAACACGGGAGCTTTGCTCTGTGGGATCAGTGGCGAACGGGTGAGTAACACGTGAGCAACCTGCCCCTGACTCTG GGATAAGCGCTGGAAACGGCGTCTAATACTGGATACGAACCACGAAGGCATCTTCAGTGGTTGGAAAGATTTATTGGTTGGGGATG GGCTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGTCGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCAC ACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCAAC GCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTTAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAAAAGCGC CGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGG TTTGTCGCGTCTGCTGTGAAATCCGGAGGCTCAACCTCCGGCCTGCAGTGGGTACGGGCAGACTAGAGTGCGGTAGGGGAGATT GGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGATCTCTGGGCCGTAACTGACGCT GAGGAGCGAAAGGGTGGGGAGCAAACAGGCTTAGATACCCTGGTAGTCCACCCCGTAAACGTTGGGAACTAGTTGTGGGGTCCAT TCCACGGATTCCGTGACGCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACG GGGACCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATATACGAGAACGGG CCAGAAATGGTCAACTCTTTGGACACTCGTAAACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC CGCAACGAGCGCAACCCTCGTTCTATGTTGCCGCANGTAATGGTGGGAACTCTTGGATACTGCCGGGGTCAACTCGGAGGAAGGG GGGATGACGTCAATCATCTGCCCCTTAG	•Microbacterium hydrocarbonoxydans strain P-SP1-2 16S ribosomal RNA gene, partial sequence, 99% query cover, 99.20% identity •Microbacterium hydrocarbonoxydans strain H21T1 16S ribosomal RNA gene, partial sequence, 99% query cover, 99.20% identity •Microbacterium sp. MDB1-30 16S ribosomal RNA gene, partial sequence, 99% query cover, 99.20% identity •Microbacterium hydrocarbonoxydans strain WR48 16S ribosomal RNA gene, partial sequence, 99% query cover, 99.20% identity •Microbacterium hydrocarbonoxydans strain NC637 16S ribosomal RNA gene, partial sequence, 99% query cover, 99.20% identity
C-B10(1)	>C-B 10 (1) | 1188 bp TCATNGCNNNNCTTACCATGCNAGTCGAACGCCCCGCAAGGGGAGTGGCAGACGGGTGAGTAACGCGTGGGAACATACCCTTTCC TGCGGAATAGCTCCGGGAAACTGGAATTAATACCGCATACGCCCTACGGGGGAAAGATTTATCGGGGAAGGATTGGCCCGCGTTG GATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGATGATCAGCCACATTGGGACTGAG ACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTG ATGAAGGCCTTAGGGTTGTAAAGCTCTTTCACCGATGAAGATAATGACGGTAGTCGGAGAAGAAGCCCCGGCTAACTTCGTGCCAG CAGCCGCGGTAATACGAAGGGGGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACGTAGGCGGATATTTAAGTCAGGGGTG AAATCCCGCAGCTCAACTGCGGAACTGCCTTTGATACTGGGTATCTTGAGTATGGAAGAGGTAAGTGGAATTCCGAGTGTAGAGGT GAAATTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTTACTGGTCCATTACTGACGCTGAGGTGCGAAAGCGTGGGGA GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGTTAGCCGTCGGGCAGTATACTGTTCGGTGGCGCAGCTA ACGCATTAAACATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCA TGTGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCTCTTGACATTCGGGGTATGGGCATTGGAGACGATGTCCTTCAGTTAG GCTGGCCCCAGAACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTC GCCCTTAGTTGCCAGCATTTAGTTGGGCCTCTAAGGNACTGCCGTGATAACCCAAGAGAAAGGTGGGATGACGTCAGTCCTCTGG CCTTACGGCTGGGCTACACNGGGCTACAGGGGGTGACGGGGCAGCAGACACNANTTCNACTATTCCAAAGCCTTCAGTCG	•Rhizobium pusense strain N39 16S ribosomal RNA gene, partial sequence 98% query cover, 97.96% identity •Agrobacterium tumefaciens strain MC7 16S ribosomal RNA gene, partial sequence 98% query cover, 97.64% identity •Rhizobium sp. strain 16161 16S ribosomal RNA gene, partial sequence 96% query cover, 98.11% identity •Agrobacterium sp. djl-8B 16S ribosomal RNA gene, partial sequence 96% query cover, 98.11% identity •Agrobacterium tumefaciens strain N70a 16S ribosomal RNA gene, partial sequence 96% query cover, 98.11% identity

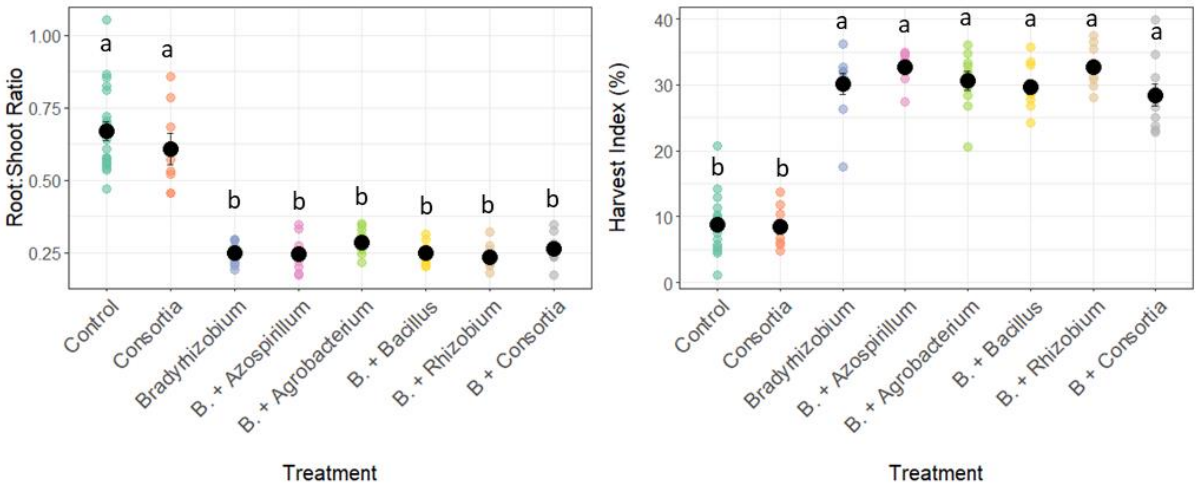

Figure S1. Root:shoot ratio and Harvest index (%) across treatments. Different letters denote Tukey post-hoc significance ($p < 0.05$) tests. Inoculation treatment had a significant impact on root: shoot ratios (ANOVA: $F_7 = 59.8$, $p < 0.001$), where overall block 2 root:shoot ratios were higher (ANOVA: $F_1 = 14.8$, $p < 0.01$). There was a significant impact of inoculation treatment (ANOVA: $F_7 = 82.3$, $p < 0.001$) on harvest index, where *Bradyrhizobium* inoculated treatments were higher.

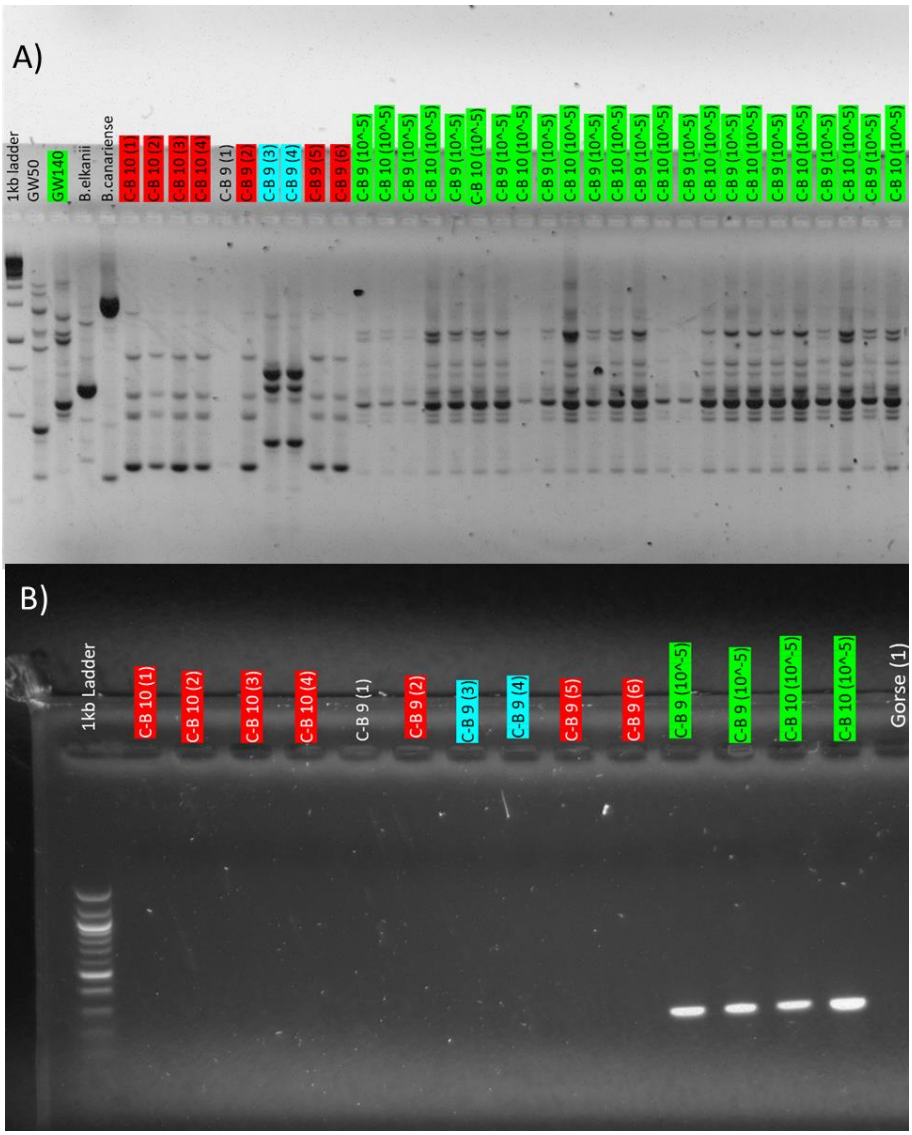

Figure S2. Gel images of A) BOXPCR profiles of nodule bacteria extracted from replicates 9 and 10 of the consortia alone treatment (labelled C-B 9 or C-B 10). Highlighted in green are BOXPCR profiles that match the GW140 BOXPCR. Highlighted in red and blue are other colonies that formed from the nodule isolates and 16S sequencing suggests they are *Agrobacterium* and *Microbacterium* isolates, respectively. B) PCR test for the presence of *Bradyrhizobium nodZ,* only colonies that proved to be *B. japonicum* were positive (highlighted in green).

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Chapter 5: Parallel molecular evolution of novel rhizobia inoculants following one soybean growing season

5.1 Abstract

Bacteria used as inoculant strains are subjected to a range of environments, of which they may be poorly adapted to. This can result in evolution of the introduced inoculant strains, which can impact the desired functional trait. Rhizobia - N_2-fixing symbionts of legumes - are inoculated alongside compatible hosts when introduced to a new range to establish biological nitrogen fixation, such as for the productive growth of soybean in the UK. Here, the potential for rhizobia inoculant evolution during the first season of soybean growth and inoculation in an agricultural setting was assessed. Field grown soybeans were sampled during the early pod fill growth stage and used to calculate %Ndfa, nodule communities were subject to shotgun sequencing. Population data was mapped to inoculant genomes (*Bradyrhizobium japonicum* SEMIA 5079 and *Bradyrhizobium diazoefficiens* SEMIA 5080) to predict mutation frequencies. Surrounding rhizosphere bacteria and *Alphaproteobacteria* communities were assessed via amplicon sequencing. Results revealed that inoculant strains were rare in the rhizosphere, but highly dominant within nodule communities, where variants were detected. Surprisingly, 47% - 57% of polymorphic variation was discovered at multiple field sites, suggesting that these mutations arose early, potentially during the inoculant production process. There were also mutations that were unique to field sites, suggesting post- introduction, site-specific selection pressures. Notably, mutations occurred in genes involved in transport and metabolism, with only one mutation evident in the symbiosis region for one for the inoculant strains (*B. diazoefficiens* SEMIA 5080). Research presented here furthers our understanding of inoculant evolution and the impact of introducing non-native rhizobia to novel environments.

5.2 Introduction

Bioinoculants are considered a more sustainable route to providing crops with essential nutrients than inorganic fertilisers (Sammauria *et al*., 2020), yet introducing biological entities to an environment can lead to unpredictable outcomes (Jack *et al*., 2021). Bacteria are used in a wide variety of inoculant products to aid plant growth (Basu *et al*., 2021), plant disease suppression (Das *et al*., 2017) or decontaminate soil pollution (Afzal *et al*., 2012). But bacteria can rapidly evolve, due to their short generation times, high mutation rates and through horizontal gene transfer (Brockhurst *et al*., 2017). Once introduced to an environment, selection pressures can shape the trajectory of bacterial variants, which can have knock-on impacts for the desired, introduced, functional trait.

Rhizobia are N_2-fixing bacterial symbionts of legume plants and have been used as bioinoculants in agriculture for over 100 years (Santos *et al*., 2019). Legume seeds are often inoculated with rhizobial symbionts during sowing to introduce or increase biological nitrogen fixation (BNF) in an area. Rhizobia live dual lifestyles, living saprotrophically in the soil until a compatible host legume is detected and the symbiosis initiates (Poole *et al*., 2018). After a molecular dialog, rhizobia intracellularly colonise plant root tissues and transform into a symbiotic state, called bacteroids. Reduced nitrogen (N) from the bacteria is exchanged for reduced carbon (C) from the plant, which requires significant changes in plant and rhizobial transport systems and metabolism (Udvardi and Poole, 2013). When rhizobia are no longer needed by the plant, bacteria are released back into the soil microbiome as their free- living states. As root nodule populations are formed from a small number of rhizobia cells (Ledermann *et al*., 2015), the infection stage creates a significant bottleneck of the initial symbiont population in the rhizosphere and a large return of successful symbiont populations back into the microbiome can drive the evolution of symbionts (Doin de Moura *et al*., 2023). The widespread natural variation of rhizobial symbionts observed in soil and root nodules is indirect evidence of differing selection pressures on rhizobia, which can occur at different life

stages beyond the symbiosis (Avontuur *et al*., 2019; Burghardt *et al*., 2019; Greenlon *et al*., 2019; Perez Carrascal *et al*., 2016; Wheatley *et al*., 2020). The functional traits, nodulation and N_2 fixation, are encoded on mobile genetic elements (MGEs) within rhizobial genomes (Wardell *et al*., 2022), which contribute to host compatibility, however, genetic factors outside of these sym-gene regions can also influence symbiosis outcomes (Tang and Capela, 2020). For example, specific genes involved with rhizosphere and root colonisation have been found to be essential pre-requisites for successful symbioses (Wheatley *et al*., 2020), and genes associated with nutrient acquisition, transport and metabolism can contribute to the competitiveness of strains in the rhizosphere (Ledermann *et al*., 2021; Mendoza-Suárez *et al*., 2021; Siqueira *et al*., 2014).

Inoculant rhizobia are more likely to experience vastly different selection pressures, as ‘elite’ strains that are isolated from one environment (*e.g.* tropical soils) are introduced to new environments (*e.g*. temperate soils), where they are likely to be poorly adapted to that environment. Therefore, we might expect inoculant strains to evolve, resulting in rapid adaptation to the novel environment, which could facilitate establishment. Additionally, the process of inoculant production is multi-faceted and requires growing strains to extremely high population densities in industrial size (*e.g.* 10,000 L) vats (Kaminsky *et al*., 2019), where usually a minimum of 10 - 20 generations are needed to achieve the required inoculant biomass (Takors, 2012). Strains may be grown separately, or together, depending on production protocols (Garcia *et al*., 2021), which could further influence inoculant composition. In these nutrient-rich, growth-unlimited conditions, mutations are more likely to arise and accumulate, particularly if populations remain at stationary phase. Exponential phase cells have been found to survive longer in liquid and soil environments, however stationary phase cells have been found to better adapt to stressors (Soria *et al*., 2006). Stress-induced responses can be activated in stationary phase cultures or during other processes such as desiccation, a step involved for seed coating inoculants (Greffe and Michiels, 2020), which may trigger increased mutation rates (MacLean, Torres-Barceló and

Moxon, 2013). Therefore, the inoculant production process may inadvertently be promoting evolution and creating diverse intraspecific inocula.

Temperate soybean cultivars are being introduced to UK agriculture and alongside them, compatible soya-nodulating rhizobia (SNR). Inoculation is essential in areas where soya is being grown for the first time as these soils do not naturally contain SNR. This provides an opportunity to study the evolution of introduced inoculants during their first season of growth. Exploring microbial evolution *in situ*, in an environmental context, is hard due to chaos and complexity of natural microbial ecosystems. Research focusing on the fate of introduced inoculants to the soil microbiome has become possible with the advancement of sequencing technologies and bioinformatics analyses (Manfredini *et al*., 2021; Mawarda *et al*., 2020). In this study, molecular evolution of the introduced SNR inoculant strains was investigated in an agricultural setting by whole population genome sequencing of field grown soybean nodules. Single nucleotide polymorphisms (SNPs), insertions and deletions (indels) were predicted by mapping population data to the inoculant genomes. The surrounding rhizosphere communities of the field grown soybeans were characterised by amplicon sequencing and the percentage of N derived from the atmosphere (%Ndfa) was quantified for soybeans during their first season of growth.

5.3 Methods

Field Sampling

Five farm sites growing soybean with seed coat inoculants for the first time in 2021 were identified in the East of England, all below the Vale of York. A list of these sites (named HF, SF, TF, NFF and GF), field crop history and soil characteristics can be found in Table S1. All sites planted the cultivar Siverka (SoyaUK) in late April/ early May 2021, that came pre-coated with the inoculant strains *Bradyrhizobium japonicum* SEMIA 5079 and *Bradyrhizobium diazoefficiens* SEMIA 5080 (Legume Technologies Ltd). In August 2021, six

replicate plants were randomly sampled from at least 20 m from the field edge within each field. Whole soybean plants with root systems and attached soil were dug up and stored at 4 ºC overnight. Additionally whole cereals plants (wheat or barley) and soil were sampled from another field on the same farm site, to check whether soya- nodulating rhizobia (SNR) were present in the soil microbiomes elsewhere on the farm. Sampling equipment was decontaminated with 70% EtOH and distilled water in between fields and sites. Bulk soil was removed from the root system by shaking and used for physicochemical analyses and isolating rhizobia. Rhizosphere soil was brushed off root systems, frozen in liquid N_2 and stored at -80 ºC before DNA extraction. Nodules were counted, removed and surface sterilised (1 minute in 70% EtOH, 4 mins in 2.5% NaOCl followed by 6 washes in sterile dH_2O). A maximum of 50 nodules were pooled per plant replicate (Table S1) and crushed in 1 mL sterilised rhizobial wash buffer solution (10 mM $MgSO_4$ and 0.01% tween solution). An aliquot removed from the crushed nodule mixture (500 µL) was frozen in liquid N_2 and stored at -80 ºC before DNA extraction. Soybean aboveground biomass was removed, dried at 80 ºC for 48 hours and used to calculate the percentage of N derived from the atmosphere (%Nfda). Three out of five field sites had enough soybean samples that were nodulated to provide replication within field sites for WGS shotgun metagenomics (Table S1), therefore only samples from those sites were used for investigating inoculant evolution (Figure 1).

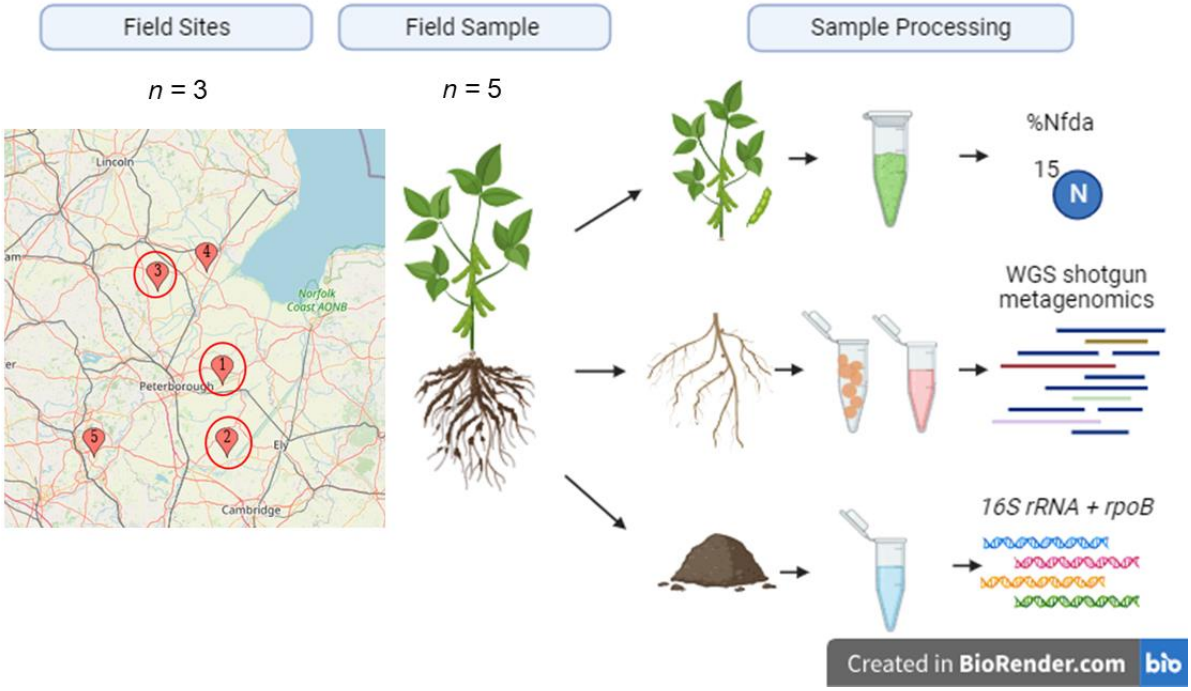

Figure 1. An overview of the field samples used for investigating inoculant evolution. Soybeans were sampled from five UK field sites growing soybean for the first time (left), where three field sites (HF, TF, NFF) had five sufficiently nodulated plant replicates (red circles). Aboveground biomass from all field sites was removed, dried and milled for %Ndfa estimation with the ^{15}N natural abundance method. Rhizosphere soil was removed from roots and DNA extracts were used for *16S rRNA* and *rpoB* amplicon sequencing of the soil microbiome from sites 1, 2 and 3. Nodules (maximum 50) were pooled per plant replicate, DNA from nodule extracts of 5 plant replicates from sites 1, 2 and 3 (*n* = 15) were used for WGS shotgun metagenomics. Created with Biorender.com.

Soil physicochemical analysis

To determine plant available nutrients in the soil, bulk soil samples were sent to Yara UK Ltd (York, UK) for quantification of phosphorus (P), magnesium (Mg), potassium (K) and pH. Briefly, P was extracted using Olsens P method and assessed using spectrophotometry, Mg and K were extracted in 1 M ammonium nitrate and assessed via atomic absorption or inductively coupled plasma analyser (ICP), pH was assessed in water. Soil moisture content was determined by standard methods listed in (Klute, 2018). For ammonium and nitrate, 10 g of soil was combined with 40 mL 2M KCL, shaken for 1 h at 200 rpm, 25°C (230VAC

Incubated Shaker, Korea), filtered through Whatman filter paper No. 42 and filtrates stored at 4 °C overnight before downstream analysis (Klute, 2018). Soil ammonium and nitrate concentrations were determined by colorimetric methods (Baethgen and Alley, 1989; Miranda *et al*., 2001) adjusted for a microtiter plate format (Tecan, SparkControl). Samples were left to incubate at room temperature for 30 minutes, (ammonium) and 2 hours (nitrate) before analysing against a standard curve.

Trapping and typing rhizobia isolates

To assess the diversity of introduced and native *Bradyrhizobium* and screen for potential horizontal gene transfer events between the two, soybean (cv. Siverka) and native legumes, gorse (*Ulex europaeus*) and broom (*Cytisus scoparius*) were grown in the bulk soil samples from both soybean and cereals fields. Four plants per field soil were planted in separate pots. Soybeans were harvested at six weeks, root nodules sterilised (as above), crushed individually and spread onto yeast mannitol agar supplement with congo red (0.025%) (Howieson and Dilworth, 2016). Gorse and broom plants were harvested at 10 weeks, due to their longer growth cycles and nodule isolates extracted (as above). Single isolates were streaked out to obtain pure cultures and boil prepped colonies were subjected to; BOXPCR, which generates a fingerprint-like strain pattern (Versalovic *et al*., 1994), a multiplex *rpoB* PCR, which gives two bands for a *B. diazoefficiens* strain (900 bp and 137 bp) and 1 band for a *B. japonicum* strain (137 bp) and *Bradyrhizobium nodZ* PCR, where a 293 bp product indicates the presence of *B. japonicum*/ *B. diazoefficiens* soya-nodulating *nodZ* (Table S2). BOXPCR can discriminate between *Bradyrhizobium* species (Fig S2; Chapter 2), patterns were checked against *B. diazoefficiens (*GW50/ SEMIA 5080) and *B. japonicum* (GW140/ SEMIA 5079) alongside the multiplex rpoB to indicate the species. *Bradyrhizobium nodZ* was conducted to check whether isolates possessed *B. diazoefficiens* / *B. japonicum nodZ* indicating the potential ability to nodulate soybean.

Calculation of BNF

Soybean plants taken from the field were sampled at early pod-fill (R3-R4) when plants were at their peak N_2 fixation stage (Ciampitti *et al*., 2021), and were used to estimate biologically fixed N using the ^{15}N Natural abundance method (Maluk *et al*., 2022; Unkovich *et al*., 2008). Adjacent non-legume dicot weeds were taken at each soybean sample location as non-fixing reference plants so each soybean replicate had a replicate weed 'reference', which should reflect plant-available soil N since these plants can only acquire N from their soil environment. Aboveground biomass was milled to a fine powder and analysed for ^{15}N and %N on an isotope ratio mass spectrometer (ANCA GSL 20-20 Mass Spectrometer; Sercon Cheshire). Delta ^{15}N values (‰) were calculated using internal standards (air) where absolute isotope ratios are measured for sample and standard and the relative measure of delta is calculated thus: $\boldsymbol{\delta}^{15}N$ (‰) vs [std] = (R_{sample} - R_{std}) / R_{std} where R = (Atom%^{15}N / Atom%^{14}N). The proportion of soybean N derived from atmospheric N_2 (%Ndfa) was calculated by comparing the $\boldsymbol{\delta}^{15}N$ of the aboveground soybean N ($\boldsymbol{\delta}^{15}N$ legume) to the average $\boldsymbol{\delta}^{15}N$ of the non-N_2-fixing reference plants ($\boldsymbol{\delta}^{15}N$ reference) and is portrayed in equation 1.

1. %Ndfa = 100 × ($\boldsymbol{\delta}^{15}N$ reference − $\boldsymbol{\delta}^{15}N$ legume) / $\boldsymbol{\delta}^{15}N$ reference − *B*

Where *B* represents the $\boldsymbol{\delta}^{15}N$ of soybeans grown entirely reliant on symbiotic nitrogen fixation. The *B* value (-0.676) used to calculate %Nfda was generated for the cv. Siverka in Chapter 4, where plants were grown in a sterile sand and vermiculite pot with no N inputs and inoculated with the two strains *B. diazoefficiens* (GW50 / SEMIA 5080) and *B. japonicum* (GW140 / SEMIA 5079). Using *B* values for the same cultivar and inoculant strains is advised for the most accurate prediction of BNF capacity of the host legume (Unkovich *et al*., 2008). Values for mg N g^{-1} plant dry weight were calculated as described in Chapter 4.

DNA Extraction and community sequencing

Rhizosphere soil and nodule samples were extracted using the Qiagen DNeasy PowerSoil Pro kit using 250 mg or 250 µL input material, respectively. Rhizosphere soil samples were used to generate PCR amplicons for the *16S* V4 region and *rpoB* gene targeting the Class *Alphaproteobacteria* (see Table S2 for primer and PCR conditions). Amplicons were sequenced 2 x 250bp on Illumina Miseq at the Centre for Genomic Research (University of Liverpool). Nodule DNA was used for shotgun metagenomics (WGS) prepared using the NEB Ultra II DNA kit and sequenced on the NovaSeq SP 2 x 150bp at the Centre for Genomic Research.

Bioinformatics analyses

For soybean rhizosphere community characterisation, *16S* and *rpoB* amplicon sequencing trimmed reads were put through the QIIME2 pipeline (v2021.11; Bolyen *et al.*, 2019) and denoised using DADA2 (v1.18; Callahan *et al.*, 2016) as previously described methods (Chapter 2). Briefly, for the taxonomic assignment for the *16S rRNA* dataset, the NCBI *16S rRNA* RefSeq database was downloaded (O'Leary *et al*., 2016), and assigned using RESCRIPt (Li *et al*., 2021) in QIIME2 with confidence values. The *Alphaproteobacteria rpoB* database curated in Chapter 2 was imported into QIIME2, a taxonomy classifier was trained using the *rpoB* primer extracted reads, and taxonomy was assigned to amplicon sequence variants (ASVs) with confidence values. Datasets were rarefied to the sample with the lowest sequencing depth, (*16S* = 260,249 sequences per sample, *rpoB* = 227,306 sequences per sample). The DNA kit negative controls contained 2 ASVs, an unclassified bacteria (4 reads) and unclassified *Actinomycetota* (3 reads) for *16S rRNA* and 2 ASVs for *rpoB*, an unclassified *Devosia* ASV (1 read) and an unclassified *Bradyrhizobium* ASV (1 read), rarefying removed these ASVs which were only present in the kit negatives and not in any samples.

For nodule community characterisation and assessing inoculant evolution, the nodule metagenomics raw fastq files were trimmed using Cutadapt (v1.2.1) followed by Sickle (v1.200) with a minimum window quality score of 20. Reads shorter than 15 bp after trimming were removed and remaining reads quality checked (FastQC). Plant host DNA was removed using Bowtie2 (v2.5.1) by mapping reads to the reference *Glycine max* Williams 82 genome (v2.1, ENA; GCA_000004515). Kraken2 was used to assess taxonomy within each sample using the standard database plus protozoa, fungi & plant capped at 8GB (Lu *et al*., 2017). Kraken2 utilises k-mer lengths and the lowest common ancestor (LCA) method to assign taxonomy and Bracken estimates relative abundance of the assigned taxonomy on read data (Lu *et al*., 2017). Abundance was calculated for reads that could be assigned at the species level. Host read removal resulted in a maximum of 7.07% of reads being assigned to the taxon *Glycine*. The flag –report-minimizer-data was added to report the number of distinct k-mers associated with each taxon classifying the sequencing reads to validate the Kraken output. Filtered reads were used for downstream bioinformatics analysis.

Mutations in inoculant genomes were predicted using Breseq v0.37.0 (Deatherage and Barrick, 2014). Breseq was originally developed to analyse the Lenski evolution experiments (Lenski, 2017) and others with select and resequence designs (Kofler and Schlötterer, 2014), to compare evolved and ancestor genomes to identify mutations and has recently been used in a rhizobial experimental evolution context (Doin de Moura *et al*., 2023). Here, Breseq is used on environmental samples to investigate genetic evolution of rhizobial inoculants *in situ.* Breseq was run using the filtered forward and reverse metagenomic reads against the reference inoculant genomes *B. japonicum* SEMIA 5079 (NCBI; NZ_CP007569), and *B. diazoefficien*s SEMIA 5080 (NCBI; NZ_ADOU02000007). The genomes used have been rotated to start at the origin of replication and SEMIA 5080 had been scaffolded to *B. diazoefficiens* USDA122 (closest relative) to create a closed genome (Weisberg *et al*., 2022b). The program was run in polymorphism mode (-p) to

predict mutation frequencies in a population. To minimise erroneous calls, the additional flag -polymorphism-minimum-coverage-each-strand was set to 20, requiring 20 forward and 20 reverse strands to have the polymorphism before it is called. Initial assessment revealed 348 mutations in 178 unique loci in *B. japonicum* SEMIA 5079 and 523 mutations in 165 unique genes in *B. diazoefficiens* SEMIA 5080, across all samples. As breseq in polymorphism mode can predict false positives (Deatherage and Barrick, 2014), final mutations lists were curated manually by checking read alignment variants with NCBI blast to check they were most closely related to the inoculant genomes and not either i) an ortholog within the same genome ii) a homolog in the other inoculant reference genome or iii) another *Bradyrhizobium* species, as *Bradyrhizobium* are a diverse clade which can be highly conserved in core regions. To prevent the possibility of including false positives and to only focus on the most prevalent mutations, an additional set of stringent rules were applied to the mutation predictions (Table 1). Additionally, for *B. diazoefficiens* SEMIA 5080, there was a large number of predicted mutations that were at similar and intermediate frequencies (~40- 60%) across all samples. When assessing read variants with NCBI Blast, some had a higher percentage identity to other *B. diazoefficiens* strains over *B. diazoefficiens* SEMIA 5080. This could highlight a potential polymorphic population introduced to the field sites, where multiple strains were present during the inoculant production process, for which we do not have the reference genomes. As these predicted polymorphisms could not be confidently assigned to the SEMIA 5080 strain they were also screened out.

Table 1. Breakdown of predicted mutations that were removed sequentially during the screening process.

Mutation Prediction	Justification for removal	No. mutations in *B. japonicum* SEMIA 5079	No. mutations in *B. diazoefficiens* SEMIA 5080
Core genes (16S & 23S)	Mutations are not expected to arise in highly conserved areas and predictions in core genes turned out to be other conserved gene regions in the community aligning e.g. soybean chloroplast or mitochondria DNA. Additionally, only present in *B. diazoefficiens* which may be due to being the first reference genome provided.	0	96
Transposable elements genes	Predictions in transposase- related genes often had multiple co-varying SNPs and aligned to multiple areas of the genomes.	50 (96% in ICE^{sym} element A)	39 (23% in ICE^{sym} element A)
Present at 100% frequency in ≥ 80% samples (probable ancestral mutation)	Some mutations returned a frequency of 100% in the samples, suggesting they are fixed in the population, these were removed if this mutation was present at 100% in 80% or more of the samples. The 80% cut off allowed for samples where nodule communities were dominated by only one inoculant strain. These mutations may represent an ancestral mutation in the inoculant genomes that most likely originated prior to introduction to these farm sites.	46	12
Low frequency mutations (≤ 10%)	Mutations that had a frequency below 10% across all samples were removed due to their low occurrence.	88	81
Screened out by cross-checking read alignment evidence with NCBI Blast	Remaining mutation evidence was checked by blasting mutated variants to check whether the inoculant genomes were most closely related. If other *B. japonicum*, *B. diazoefficiens* or other *Bradyrhizobium* strains had a higher percentage identity to the mutated variant, these mutations were removed and may reflect variants already present on the seed coat or in the soil microbiome.	106	262

Data analysis

Data was analysed on R (v4.3; R Core Team, 2022) with Rstudio (R Studio Team, 2020). Amplicon sequencing *16S rRNA* and *rpoB* rarefied datasets were analysed with the package 'phyloseq' (McMurdie and Holmes, 2013) and 'vegan' (Oksanen *et al*., 2020). BNF and soil traits were analysed with packages from tidyverse, non-parametric tests such as the Kruskal-Wallis test was used to assess whether there were significant differences between sites for rank data (%Ndfa and alpha- diversity metrics). PERMANOVA using 'adonis2' with 999 permutations was used for assessing whether field sites varied for beta- diversity (Bray-Curtis Distances), after checking the homogeneity of variances. The *Alphaproteobacteria rpoB* dataset was subsetted to the reads assigned to the *Bradyrhizobium* genus to investigate dynamics in the *Bradyrhizobium* community. The shotgun metagenomics kraken2 taxonomic assignments and bracken abundance data was imported into R and analysed with 'phyloseq' for microbiome related measures, using the above methods.

Go term analysis was performed on the subsetted list of genes with mutations and compared to the inoculant genomes to see if any specific functions were being targeted. Gene Ontology (GO) terms for the inoculant genomes were downloaded from UniprotKB (The UniProt Consortium, 2023) and GO enrichment analysis was performed with the R package topGO (Alexa and Rahnenfuhrer, 2023). Only unambiguous proteins that had been previously assigned GO terms were considered (removing all mutations in hypothetical genes). If a mutation occurred in an intergenic region, both genes were included in the GO analysis. Two algorithms were tested, classic (which does not take the GO hierarchy into account) and weight01 (a combination of 'elim' and 'weight' algorithms which takes a bottom up approach and compares parent- child scores to find the locally most significant GO term, respectively; Alexa and Rahnenfuhrer, 2020). Fisher's Exact tests with the weight01 algorithm were conducted to determine if any GO terms were significantly enriched. A Bonferroni false discovery rate was calculated (Jafari and Ansari-Pour, 2019), (0.05 / number of GO terms assessed), two GO terms had lower p- values than the threshold values, however as correcting for multiple testing using an algorithm that takes into account GO hierarchy (weight01) can be overly stringent, GO terms with significance to $p< 0.05$ (unadjusted) are reported (Alexa and Rahnenfuhrer, 2020).

To predict whether mutations in proteins would be tolerated or deleterious, the web tool SIFT was used (Sim *et al*., 2012), scores range from 0 - 1 with deleterious amino acid changes predicted for scores < 0.05. Sift scores are only presented if below 0.05. To identify whether mutations occurred within certain protein domains, the web tool Interpro was used (Paysan-Lafosse *et al*., 2023). A bootstrapping simulation test was written in base R (v4.3; R Core Team, 2022) with Rstudio (R Studio Team, 2020) to test if mutations were expected to occur within the ICE symbiosis elements by chance. The function performed 1000 simulations and returned the number of simulations where no randomly generated mutations were within the ICE^{sym} A or B elements.

5.4 Results and Discussion

SNR only present in fields where introduced

To confirm that there were no native SNR present at the field sites, soybean was grown in the soil sampled from soya and cereal fields from the five sites and examined for nodulation after five weeks. No plants grown in the cereals soil had nodules (Figure S1), indicating that there were no native SNR present on the farm sites. In contrast, all soybeans grown in the soya soil from all sites were nodulated. Isolates were found to be *B. diazoefficiens* or *B. japonicum* through a combination of BOXPCR, Bj/ Bd multiplex PCR and presence of *Bradyrhizobium japonicum/ diazoefficiens nodZ. Bradyrhizobium* isolated from the soya soils were mainly *Bradyrhizobium diazoefficiens,* whereas *Bradyrhizobium japonicum* was the dominant symbiont at only 1 site (NFF) out of the 5 sites (Table 1). Four isolates per site (or three for site SF) had the 283bp *rpoB* region sequenced, using primers without red overhangs in Table S2, and confirmed 100% matches to either inoculant strain.

Table 2. *Bradyrhizobium* isolates extracted from individual soybean nodules grown as trap plants in soya and SNR introduced soil (*n* = 4).

Farm Site	No. isolates *B. diazoefficiens*	No. isolates *B. japonicum*
SF	2	1
GF	19	0
HF	26	0
NFF	1	16
TF	17	4

Native legumes gorse (*Ulex europaeus*) and broom (*Cytisus scoparius*) were also grown in the cereals soil and soya soil to trap any native *Bradyrhizobium* isolates. After 10 weeks of growth, only broom plants in GF and SF cereals soil and broom plants grown in HF soya soil had root nodules, of which there were very few and were white-ish in colour,

indicating that they were not fixing N_2. Isolates extracted from the white nodules showed the highest sequence similarity (*rpoB*) to *Rhizobium leguminosarum* species (Table S3), which was unexpected as broom is usually nodulated by *Bradyrhizobium* sv. *genistae* (Stępkowski *et al*., 2018). *R. leguminosarum* genospecies are abundant in UK agricultural soils (Maluk *et al*., 2022).

%Ndfa varied across field sites

The percentage of N derived from the atmosphere (%Ndfa) in field grown soybeans varied significantly between farm sites on their first season of growth (Kruskal-Wallis χ^2 = 17.798, df = 4, p < 0.01, see Figure 2). Values ranged from an average of 25.1% Ndfa at site GF to 47.8% at site HF. Nodule frequency reflected %Ndfa, which was lowest in GF and highest in HF, however a Kruskal- Wallis test between sites was not significant (Figure 2). Correlations between soil nutrients and plant N were explored as various environmental factors can impact BNF levels, there was a significant positive correlation between soil P and plant mg N g^{-1} (r = 0.64, *n* = 30, p < 0.001) and a significant negative correlation between soil moisture content (SMC) and %Ndfa (r = -0.41, *n* = 30, p < 0.05; Figure S2).

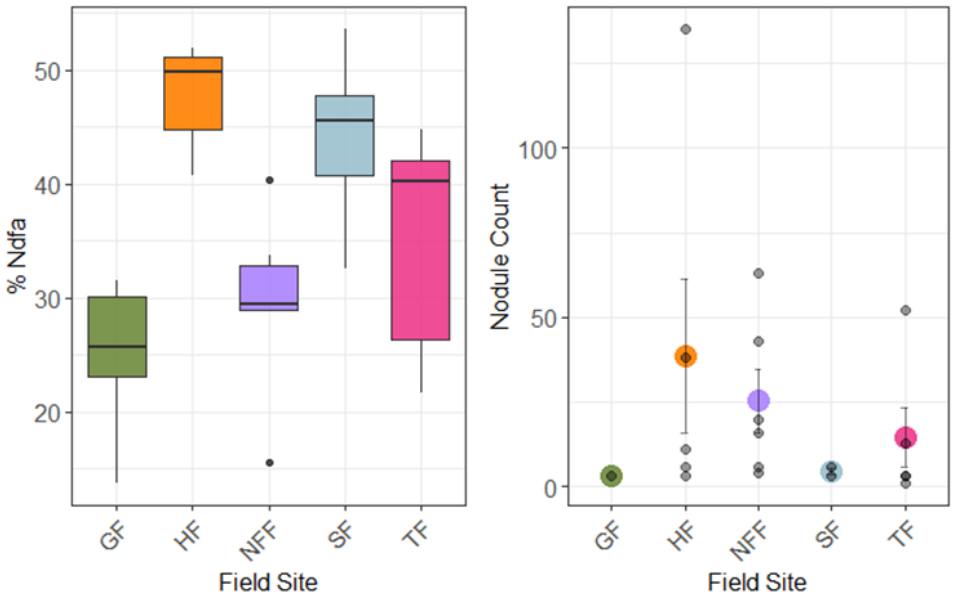

Figure 2. Percentage of Nitrogen derived from the atmosphere (%Ndfa) in soybeans from five UK

field sites calculated using the ^{15}N natural abundance method (*n* = 6). Nodule count from UK field grown soybean samples. Large coloured circles represent the average, smaller grey circles represent individual replicates, error bars represent the standard error (*n* = 6).

Soya rhizosphere communities are characterised by site specific variation, where inoculant species are rare

Three field sites (HF, NFF, TF) had five plant replicates with sufficient nodule material for metagenomics sequencing. The surrounding rhizosphere communities of these nodule samples were sequenced via amplicon sequencing (refer to Figure 1). Amplicon sequencing of the *16S rRNA* and *rpoB* gene targets for the surrounding soya rhizosphere samples resulted in 14,839 ASVs and 7,569 ASVs, respectively after DADA2 denoising and rarefaction.

Rhizosphere communities sampled from the same soybean cultivar across the 3 different field sites significantly differed in their level of diversity and composition at the bacterial community level, *Alphaproteobacteria* class level and at the genus *Bradyrhizobium* level (Figure 3). Alpha diversity, varied in the soya rhizospheres across the 3 sites for the whole bacterial community (Shannon's: Kruskal-Wallis χ^2 = 8.64, df = 2, p-value < 0.05; Simpson's: Kruskal-Wallis χ^2 = 9.42, df = 2, p-value < 0.01; Figure 3A), *Alphaproteobacteria* community (Shannon's: Kruskal-Wallis χ^2 = 7.76, df = 2, p-value < 0.05; Simpson's: Kruskal-Wallis χ^2 = 6.5, df = 2, p-value < 0.05; Figure 3D), and *Bradyrhizobium* community (Simpson's: Kruskal-Wallis χ^2 = 6.5, df = 2, p-value < 0.05; Figure 3G) where site HF had higher community diversity and evenness and site NFF had the lowest community richness and evenness, suggesting a higher dominance of one or a few members of the community in this soil. Rhizosphere soil microbial community composition (Bray- Curtis distances) also significantly differed between sites for the for the *16S* bacterial community (PERMANOVA, F = 10.66, R^2 = 0.64, p< 0.001; Figure 3B), *rpoB Alphaproteobacteria* community (PERMANOVA, F = 19.13, R^2= 0.76, p< 0.001; Figure 3E) and for the genus *Bradyrhizobium*

(PERMANOVA, F = 19.42, R^2 = 0.76, p< 0.001; Figure 3H), with clear separation by field site.

There is a diverse resident *Bradyrhizobium* community in the rhizosphere and the inoculant species do not dominate these communities (Figure 3I). *Bradyrhizobium diazoefficiens* were rare in the soil microbiomes and only detected in samples HF_1 (0.7% relative abundance), TF_2 (0.3%) and TF_5 (0.2%) and not at all at site NFF. *Bradyrhizobium japonicum* was detected in three out of five samples from site HF ranging in relative abundance 0.16% - 42% , all samples from site NFF (0.03% - 0.38%) and only one sample from site TF (0.2%). There is an exception in sample HF_2, where *Bradyrhizobium japonicum* is at high relative abundance (42%). This plant sample also exhibited the highest frequency of nodules on root systems (135). Relative abundance of taxonomic phyla from the *16S* dataset and *Alphaproteobacteria* genera from the *rpoB* dataset can be seen in Figure 3C and 3F. These results suggest that the inoculant species are not highly dominant in the rhizosphere communities during the pod- fill plant growth stage.

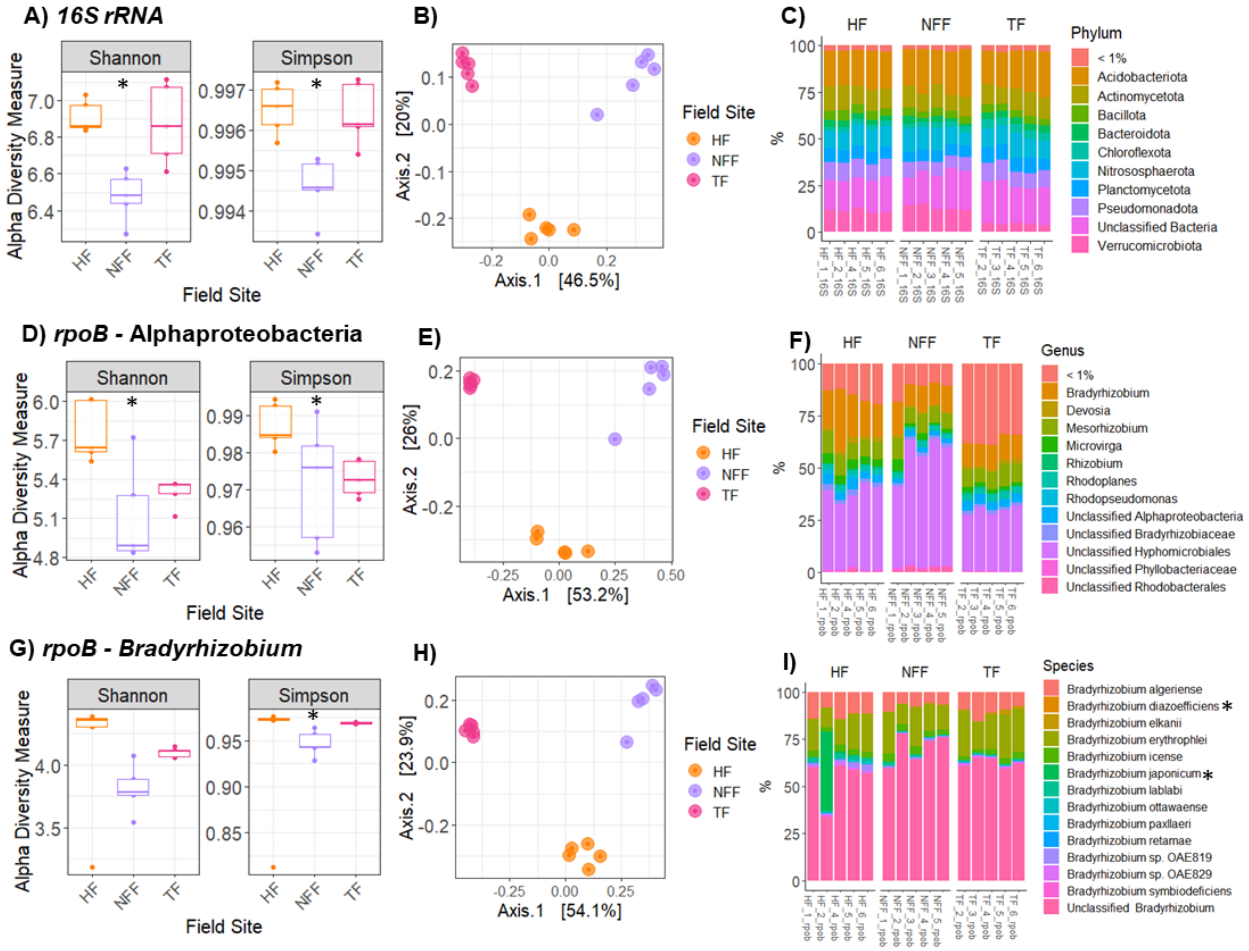

Figure 3. Microbiome metrics for soybean rhizosphere communities sampled across 3 UK field sites (HF, NFF, TF, *n* = 5). **A)** Alpha- diversity measures Shannon's and Simpson's Index of soil *16S rRNA* dataset, black star denote statistical significance by Kruskal- Wallis test. **B)** Principal coordinates analysis (PCoA) of Bray- Curtis dissimilarity matrix for *16S*. **C)** Relative abundance of taxonomic Phyla for *16S*. **D)** Alpha- diversity measures Shannon's and Simpson's Index of soil *rpoB* (*Alphaproteobacteria*) dataset, black star denote statistical significance by Kruskal- Wallis test. **E)** PCoA of Bray- Curtis dissimilarity matrix for *Alphaproteobacteria rpoB*. **F)** Relative abundance of taxonomic genera for *rpoB*. **G)** Alpha- diversity measures Shannon's and Simpson's Index of soil *rpoB* amplicon dataset subsetted to the genus *Bradyrhizobium,* black star denote statistical significance by Kruskal- Wallis test. **H)** PCoA of Bray- Curtis dissimilarity matrix for *Bradyrhizobium rpoB*. **I)** Relative abundance of *Bradyrhizobium* species assigned by *rpoB*, inoculant species highlighted with black star (*).

Inoculant species dominate nodule communities

The inoculant species were highly dominant in the nodule communities as expected, but other *Bradyrhizobium* species were also present in very low abundance (Figure 4). Within the nodule metagenomics communities, 437 taxa were assigned to the species level using kraken2 and bracken species abundance estimation (Lu *et al*., 2022). Of these 437 taxa, 326 were assigned to the Kingdom Bacteria, 109 to Eukaryota (93% Streptophyta plant DNA, 6.5% Ascomycota Fungal DNA) and 2 were assigned to Viruses. The most diverse and abundant genus was *Bradyrhizobium* with 73 species described, of which *B. japonicum* and *B. diazoefficiens* were highly dominant (>99%, Figure 4C). There was no significant difference between sites based on alpha (Figure 4A), or beta diversity (Bray- Curtis dissimilarity; Figure 4B). There was variation amongst plant replicates: at site TF and HF there is evidence of both inoculant strains capable of becoming the dominant symbiont within plant replicates (HF_1, HF_5, TF_6, TF_5), whereas at site NFF, *B. japonicum* is at higher abundance across the replicates and dominated one replicate (NFF_5). Notably, *B. japonicum* isolates were the dominant symbiont isolated from the NFF soya soil trap plants (refer to Table 2). Among the remaining <1% of nodule diversity, the next most abundant taxa were other *Bradyrhizobium* species, which were present at very low relative abundances and were shared across all sites (Figure 4D).

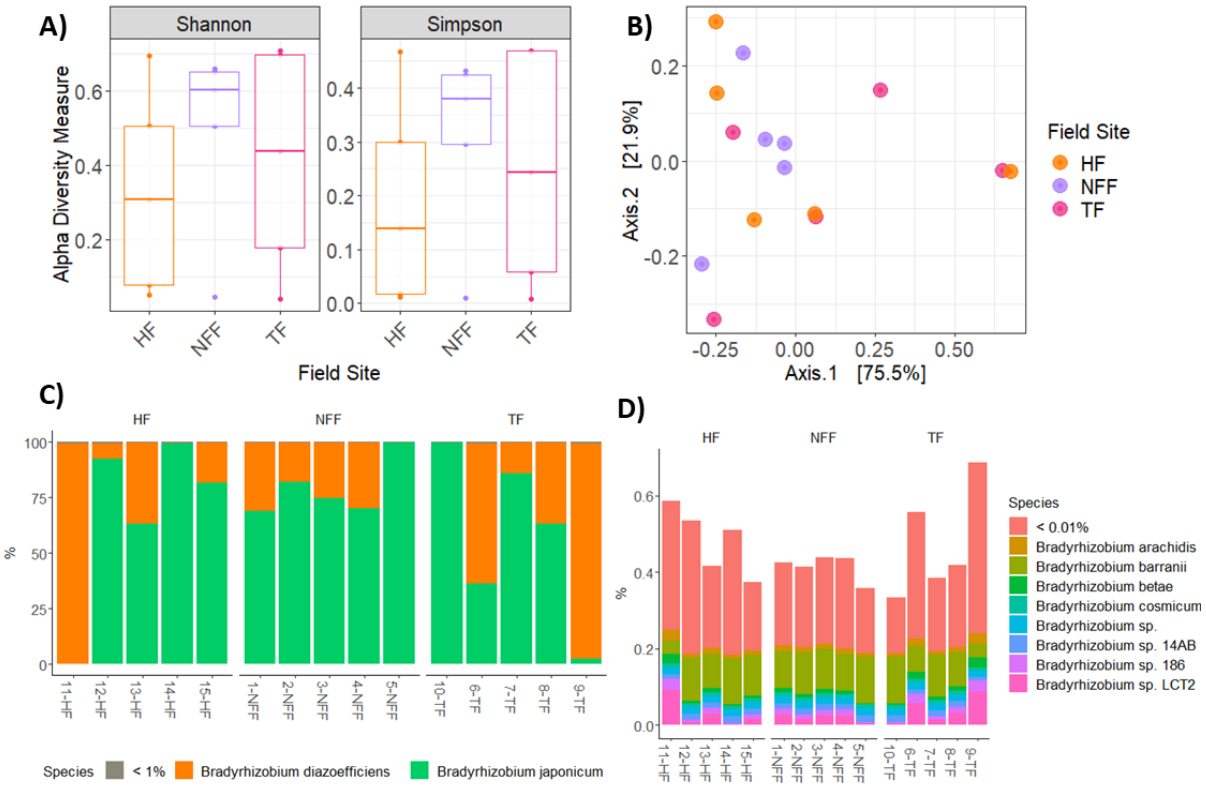

Figure 4. Microbiome metrics for nodule communities from 3 UK field sites (HF, NFF, TF; *n* = 5). **A)** Alpha- diversity measures Shannon's and Simpson's Index. **B)** PCoA of Bray- Curtis dissimilarity matrix **C)** Relative abundance of inoculant species in nodules. **D)** Relative abundance of non-inoculant species above 0.01% abundance in nodules (a zoomed in visual of < 1% category in graph C).

Widespread presence of inoculant mutations within nodule environments

As no native SNR were isolated from the field sites, we assume variants within nodules are direct descendents from the inoculants and Breseq was used to predict mutations. After a stringent screening process (Table 1), there were 58 mutations in 57 unique loci in *B. japonicum* and 32 mutations in 32 loci in *B. diazoefficiens* (Figure 5). In *B. japonicum*, which had significantly more mutations than *B. diazoefficiens* (χ^2 = 7.5, df = 1, p< 0.01), there was 1 nonsense, 3 frameshift, 21 nonsynonymous, 24 intergenic and 9 synonymous mutations. In *B. diazoefficiens*, there were 5 frameshifts, 14 nonsynonymous, 7 intergenic and 6

synonymous mutations. The majority of predicted mutations were single nucleotide polymorphisms (SNPs). As increasing evidence suggests that synonymous mutations may not be silent due to differences in codon usage bias (Callens *et al*., 2021; Liu, 2020) and recent evidence from rhizobial experimental evolution studies suggested a third of adaptive mutations were synonymous (Doin de Moura *et al*., 2023), both synonymous and intergenic polymorphisms are discussed and included in the analysis.

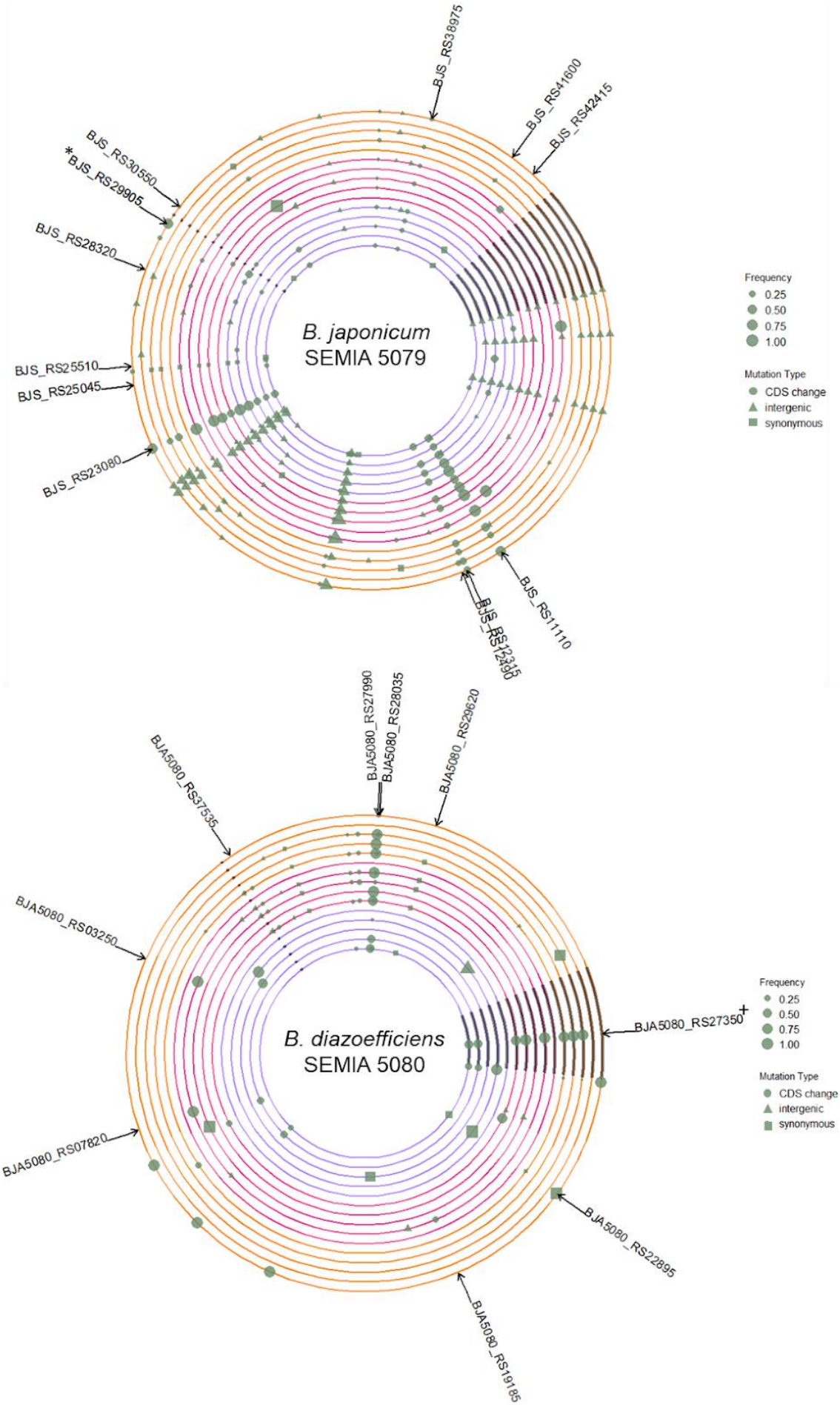
BJS_RS38975
BJS_RS41600
BJS_RS42415
BJS_RS30550
*BJS_RS29905
BJS_RS28320
B. japonicum
SEMIA 5079
BJS_RS25510
BJS_RS25045
BJS_RS23080
BJS_RS11110
BJS_RS12315
BJS_RS12390
Frequency
0.25
0.50
0.75
1.00
Mutation Type
CDS change
intergenic
synonymous
BJA5080_RS27990
BJA5080_RS28035
BJA5080_RS29620
BJA5080_RS37535
BJA5080_RS03250
B. diazoefficiens
SEMIA 5080
BJA5080_RS27350
BJA5080_RS07820
BJA5080_RS22895
BJA5080_RS19185
Frequency
0.25
0.50
0.75
1.00
Mutation Type
CDS change
intergenic
synonymous

Figure 5. Mutation frequencies within *B. japonicum* SEMIA 5079 (top) and *B. diazoefficiens* SEMIA 5080 (bottom) genomes, represented as proportions. Each replicate ring represents the length of the genome and the positions of mutations found within that nodule community. Replicates coloured by field site, purple = NFF (inner), pink = TF (middle) and orange = HF (outer). Shape corresponds to mutation type, circle = mutations resulting in changes in CDS (nonsynonymous, nonsense, frameshift mutations), triangle = intergenic mutations, square = synonymous substitutions. Size of the point denotes frequency of mutation within the population. Locus tags of mutations discussed within text are labelled, black star (*) denotes gene targeted by parallel selection, black plus sign (+) denotes genes within ICE^{sym} elements. Black regions indicate positions of tRNA-Val symbiosis ICE elements, larger symbiosis A element on the right, smaller B element on left (as predicted by Weisberg, Sachs and Chang, 2022).

Enriched functions across inoculant genomes targeted by mutations

GO analysis was performed to compare the subset of genes affected by mutation to the background of all genes within inoculant genomes, to investigate whether genes with similar functions are more likely to be targets of mutation.

B. japonicum SEMIA 5079

Two GO terms for biological processes in *B. japonicum* SEMIA 5079 passed the Bonferroni false discovery rate for multiple testing, both of which were associated with aromatic amino acid degradation, namely L-phenylalanine catabolism and tyrosine catabolism (p = <0.001, Table 3). This corresponds with the significant molecular functions homogentisate 1,2-dioxygenase (HmgA) activity and fumarylacetoacetase (FahA) activity (Table 3) which are involved in the utilisation of L-phenylalanine and tyrosine as N and C sources by rhizobia (da Silva Batista *et al*., 2010; Dunn, 2015). Amino acid degradation via HmgA is associated with N starvation in *S. meliloti* (Capela *et al*., 2006). Other functions involved in C cycling were also significant when not accounting for false discovery rate (*i.e.* $p < 0.05$, see discussion in

methods). This includes gluconeogenesis and the glycerol metabolic processes, as well as the molecular functions fructose 1,6-bisphosphate 1-phosphatase activity and methylmalonate-semialdehyde dehydrogenase (acylating) activity ($p < 0.05$; Table 3). The fructose 1,6-bisphosphate 1-phosphatase enzyme (GlpX) is essential in the conversion of glucose to pyruvate and methylmalonate-semialdehyde dehydrogenase is involved in myo-inositol metabolism, one of the most abundant plant derived compounds within soybean nodules (Vauclare *et al*., 2013). Both of these enzymes have been found to be expressed and important in bacteroid metabolism in the soybean- *Bradyrhizobium* symbiosis (Nomura *et al*., 2010; Sarma and Emerich, 2005). Riboflavin biosynthesis process is affected ($p < 0.05$), corresponding with the molecular function 6,7-dimethyl-8-ribityllumazine synthase activity ($p < 0.05$) and the riboflavin cellular component ($p < 0.05$; Table 3). This enzyme is involved in the penultimate step in riboflavin (vitamin B_2) biosynthesis, which is the precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), essential cofactors for a wide variety of redox enzymes (Riviezzi *et al*., 2021). Riboflavin availability has been found to influence rhizosphere survival and root colonisation in *R. leguminosarum bv. viciae* (Wheatley *et al*., 2020). Additionally, proton-transporting ATPase activity was significant ($p < 0.05$), alongside the cellular component proton transporting ATP synthase complex ($p < 0.05$; Table 3).

Table 3. Significant GO terms ($p < 0.05$) in *B. japonicum* SEMIA 5079. Raw *p*-values reported, highlighted in bold are GO terms that passed a false discovery rate (Bonferroni) for multiple testing.

B. japonicum SEMIA 5079					
Biological Process					
GO.ID	**Term**	**Annotated**	**Significant**	**Expected**	**weight01Fisher p-value**
GO:0006559	**L-phenylalanine catabolic process**	**2**	**2**	**0.01**	**2.30E-05**
GO:0006572	**tyrosine catabolic process**	**2**	**2**	**0.01**	**2.30E-05**
GO:0006094	gluconeogenesis	6	1	0.03	0.03
GO:0006071	glycerol metabolic process	6	1	0.03	0.03
GO:0009231	riboflavin biosynthetic process	7	1	0.04	0.035
GO:0032259	methylation	69	2	0.35	0.045
Molecular Function					
GO:0004411	homogentisate 1,2-dioxygenase activity	1	1	0.01	0.0052
GO:0004334	fumarylacetoacetase activity	1	1	0.01	0.0052
GO:0000906	6,7-dimethyl-8-ribityllumazine synthase activity	2	1	0.01	0.0104
GO:0004491	methylmalonate-semialdehyde dehydrogenase (acylating) activity	2	1	0.01	0.0104
GO:0015416	ABC-type phosphonate transporter activity	3	1	0.02	0.0156
GO:0042132	fructose 1,6-bisphosphate 1-phosphatase activity	3	1	0.02	0.0156
GO:0003908	methylated-DNA-[protein]-cysteine S-methyltransferase activity	5	1	0.03	0.0259
GO:0046961	proton-transporting ATPase activity, rotational mechanism	5	1	0.03	0.0259
Cellular Component					
GO:0009349	riboflavin synthase complex	2	1	0.01	0.012
GO:0045261	proton-transporting ATP synthase complex, catalytic core F(1)	5	1	0.03	0.031

B. diazoefficiens SEMIA 5080

In *B. diazoefficiens*, no GO terms were significant at the calculated Bonferroni false discovery rates, however the biological processes involved in cell shape regulation, cell division, cell cycle and cell wall organisation and regulation of intracellular signal transduction were significant to $p < 0.01$ (Table 4). Gene targets associated with several functions known to be linked to bacteroid activity were also found to be significantly enriched: The GMP biosynthetic process ($p < 0.01$), and the corresponding molecular function IMP dehydrogenase activity ($p < 0.01$), involved in generating guanine. Nucleotides are actively metabolised by bacteroids, and an increase in nucleosides and nucleotides has been observed in soybean - *Bradyrhizobium* nodules (Delmotte *et al*., 2010; Vauclare *et al*., 2013; Lardi *et al*., 2016). The molecular function methionine synthase ($p < 0.01$), where components of this pathway have been shown to be essential for *R. etli* nodulation of *Phaseolus vulgaris* (Taté *et al*., 1999); phosphoglucosamine mutase activity ($p < 0.01$) previously suggested to be involved for effective nod factor production in *B. elkanii* (Cooper

et al., 2018). The peptidoglycan biosynthetic process ($p < 0.05$) corresponding with the molecular functions UDP-N-acetylmuramate dehydrogenase (MurB) activity ($p< 0.01$) and UDP-N-acetylmuramate-L-alanine ligase (MurC) activity ($p < 0.01$). The molecular function acetate-CoA ligase (ADP-forming) activity ($p < 0.05$). This process results in the formation of acetyl-CoA, an essential component for C metabolism via the tricarboxylic acid (TCA) cycle, lipid and poly-hydroxybutyrate (PHB) synthesis, an intracellular C storage polymer, suggesting altered activity could have widespread impacts in N_2 fixing bacteroids (Cooper *et al*., 2018; Ledermann *et al*., 2021; Udvardi and Poole, 2013). The molecular function cobalamin binding was enriched ($p < 0.05$), which has been found to be important in rhizosphere and root colonisation for *R. leguminosarum bv. viciae* (Wheatley *et al*., 2020) and FAD binding ($p < 0.05$), an essential cofactor which acts as electron donors or acceptors in a variety of reactions, *e.g*. the TCA cycle results in vast amounts of reduced electron carriers (Ledermann *et al*., 2021).

Table 4. Significant GO terms ($p < 0.05$) in *B. diazoefficiens* SEMIA 5080. Raw p-values reported.

B. diazoefficiens SEMIA 5080					
Biological Process					
GO.ID	**Term**	**Annotated**	**Significant**	**Expected**	**weight01Fisher p-value**
GO:0008360	regulation of cell shape	21	2	0.09	0.0033
GO:0051301	cell division	28	2	0.12	0.0058
GO:0007049	cell cycle	29	2	0.12	0.0063
GO:0071555	cell wall organization	29	2	0.12	0.0063
GO:1902531	regulation of intracellular signal transduction	2	1	0.01	0.0085
GO:0006177	GMP biosynthetic process	2	1	0.01	0.0085
GO:0009252	peptidoglycan biosynthetic process	46	2	0.20	0.0153
Molecular Function					
GO:0008705	methionine synthase activity	1	1	0.00	0.0043
GO:0008966	phosphoglucosamine mutase activity	1	1	0.00	0.0043
GO:0003938	IMP dehydrogenase activity	1	1	0.00	0.0043
GO:0008762	UDP-N-acetylmuramate dehydrogenase activity	1	1	0.00	0.0043
GO:0008763	UDP-N-acetylmuramate-L-alanine ligase activity	1	1	0.00	0.0043
GO:0043758	acetate-CoA ligase (ADP-forming) activity	5	1	0.02	0.0211
GO:0031419	cobalamin binding	7	1	0.03	0.0294
GO:0050660	flavin adenine dinucleotide binding	124	3	0.53	0.0407

Distribution of polymorphisms across sites

Overall, most polymorphisms were present in more than one field site, with fewer unique to location (Figure 6). Particularly for *B. japonicum,* where 57% of mutations were present at multiple field sites, whereas 47% were present at multiple sites for *B. diazoefficiens* SEMIA 5080 (*i.e.* inner circles in Figure 6). Mutations present at multiple field sites could either be due to mutations appearing prior to inoculation on field, *e.g.* during inoculant production, or arising independently, in parallel at each field. As high rates of nucleotide level parallelism is rare, it is more likely that mutations arose early, during production and have been introduced to all sites. Once introduced, certain alleles have persisted to different frequencies within the environment. Variation in site frequencies may suggest different selective pressures. For example, the same polymorphism is sometimes present at two sites but not all three, *e.g*. a predicted tolerated missense SNP in an AraC transcriptional regulator in *B. diazoefficiens* (BJA5080_RS26170) was present at average frequencies of 27.9% at site HF, 37% at site NFF, but absent at site TF. This could suggest some alleles may be competitive at some sites, but not at others.

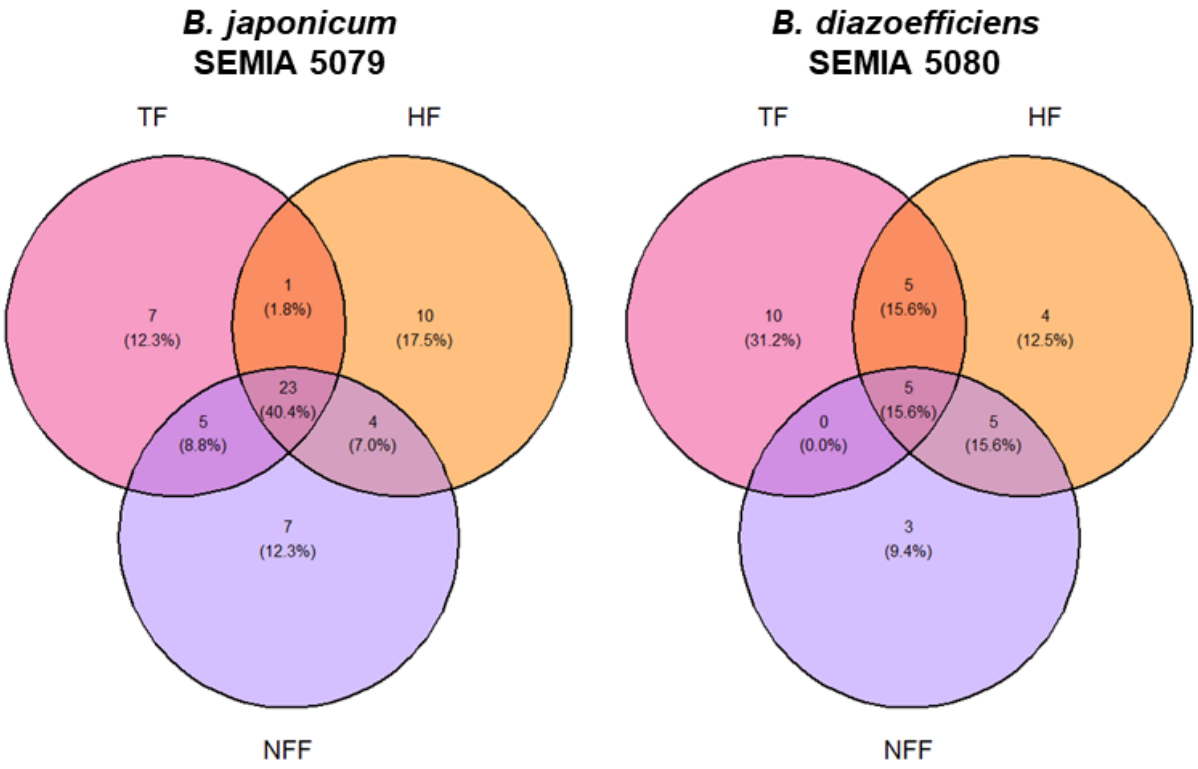

Figure 6*:* Venn diagram of the number of unique loci with mutations in inoculant genomes unique to and present at multiple field sites.

Polymorphisms present at multiple field sites

Polymorphisms that were present in more than one field site may have arisen early and could be the result of diversification prior to field site introduction. There is evidence that the introduced polymorphisms are present within the nodule communities of different field sites to varying degrees (Table 5A and 5B), suggesting the competitiveness of polymorphisms may vary across sites. There were some notable polymorphisms present across sites in genes involved with transport and metabolism.

There is evidence of polymorphisms varying in frequency in ATP-binding cassette (ABC) transporter proteins in both inoculant strains across field sites. In *B. japonicum*, a synonymous polymorphism in an ABC transporter permease (BJS_RS41600) was observed at low average frequency in site HF (2.1%) and NFF (7.2%) but not at site TF (Table 5A). There is also a tolerated missense polymorphism that occurs at low average relative frequency in an ABC transporter substrate- binding protein (BJS_RS28320) at site NFF (2.4%) and TF (1.9%) but not in site HF (Table 5A). In *B. diazoefficiens*, a deleterious missense polymorphism (SIFT: 0) in an amino acid ABC transporter substrate-binding protein (BJA5080_RS27990) is at relatively high frequencies at site HF (68.8%) and TF (68.1%) but lower at site NFF (24%; Table 5B). Additionally, in *B. diazoefficiens*, a tolerated missense polymorphism was also found in an ABC transporter permease (BJA5080_RS28035) at HF (7.7%) and TF (8.2%) and NFF (4.5%) and a synonymous polymorphism in an ABC transporter ATP-binding protein (BJA5080_RS22895) at 26.8% average frequency in HF, 31.6% in NFF but not present at site TF (Table 5B). Rhizobia genomes encode a vast amount of ABC transporter proteins and in particular 7.8% of *B. japonicum* SEMIA 5079 and 8.6% of *B. diazoefficiens* SEMIA 5080 genomes are predicted

to be ABC transporters (Siqueira *et al*., 2014). An abundance of ABC transporters have been found to be expressed in soybean bacteroids (Sarma and Emerich, 2005) and are necessary for nodule functioning (Wheatley *et al*., 2020), but are also important in the free- living state and may contribute to the saprophytic capability of *Bradyrhizobium* species. In *B. japonicum*, there is also a tolerated missense polymorphism that occurs in a PepSY domain protein (BJS_RS11110), which showed 100% similarity to FsrB, a siderophore utilisation protein. This polymorphism is at relatively high frequency particularly at site TF (63.8%) and HF (53.1%), but lower at site NFF (27%). FsrB is required to remove iron from siderophores, which allows uptake into the cytoplasm, experimentally induced mutations in *fsrB* resulted in gain of function, where a wider range of siderophores can be recognised by the protein (Ong and O'Brian, 2023). There is also a predicted deleterious missense polymorphism (SIFT: 0) in a hybrid sensor histidine kinase/response regulator (BJS_RS23080) in *B. japonicum* which occurs again at relatively high frequency at site TF (61.4%) and HF (51.5%) and lowest at NFF (25.6%). Histidine kinases are often involved in signal transduction and response to external stressors (Wülser *et al*., 2022).

There are polymorphisms in genes involved in metabolic processes across sites. In *B. japonicum*, a predicted deleterious missense polymorphism (SIFT: 0) in *phaA,* an acetyl-CoA acetyltransferase (BJS_RS38975) which is involved in both the TCA cycle and PHB synthesis is found across all three field sites at low average frequencies (3.5% - 9%; Table 5A). An increase in enzymes involved in the TCA cycle has been observed in bacteroids, but it is heavily O_2 limited, thus PHB formation often occurs under low O_2 conditions, when acetyl-CoA is metabolised to PHB (Poole *et al*., 2018). Additionally a deleterious missense polymorphism (SIFT: 0) in fructose-bisphosphatase class II (BJS_RS12315), involved in producing glycolytic intermediates (Sarma and Emerich, 2006, 2005), is evident at similar frequencies across all sites (21%- 23.9%; Table 5A). There is a tolerated missense polymorphism in an acyl-CoA dehydrogenase gene in both *B. japonicum* (BJS_RS30550; Table 5A) found across all sites (1.3%- 2.2% average frequency), and *B. diazoefficiens*

(BJA5080_RS37535; Table 5B), found at sites HF (2.3%) and TF (7.4%). Interestingly, in both proteins (which are 96% similar) the predicted amino acid change occurs at the same position (178) from a Serine to an Arginine in both inoculant strains, which may suggest selection on this gene across both inoculant species. Acyl-CoA dehydrogenases are involved in lipid fatty acid metabolism, a process found to be significantly repressed in soybean bacteroids compared to their free- living states (Sarma and Emerich, 2006).

While the majority of loci targeted across sites were by the same mutation, in one instance the same gene was targeted by different mutations at different field sites. Different mutations are more likely to have arisen independently and so can be a signature of strong selection on that locus. In the locus, BJS_RS29905, at site NFF there is a low occurrence polymorphism (7% average frequency), resulting in a predicted deleterious missense mutation (SIFT: 0.01) in a GGDEF-domain containing protein (Figure 5A, Table 5A). Whereas at sites HF and TF this locus (BJS_RS29905) has a polymorphism also predicted to be deleterious (SIFT: 0) at a different position (8038025), that occurs within the GGDEF domain at an allele frequency of 14% at site NFF and 2% average frequency at site TF (Figure 5A, Table 5A). Both of these mutations are predicted to cause loss of protein function. This gene produces a EAL-domain coding protein (NCBI protein accession no: WP_014492408.1) with dual diguanylate cyclase/ phosphodiesterase activity. This protein is associated with the nucleotide-based second messenger cyclic diguanosine monophosphate (c-di-GMP), which is involved in signal transduction (Gao *et al*., 2014). Additionally, at site TF, there is a SNP 265 bp upstream of a PilZ domain- containing protein (BJS_RS13100; Table 6A), a known c-di-GMP sensor (Amikam and Galperin, 2006). Interestingly, in the other inoculant *B. diazoefficiens,* there is a synonymous polymorphism in a diguanylate phosphodiesterase (BJA5080_RS29620) present across all field sites (Table 5B) and a predicted deleterious missense polymorphism (SIFT: 0) in a diguanylate cyclase gene (BJA5080_RS03250) which swept to fixation (100%) in one plant replicate at field site TF (Table 6B). These mutations potentially suggest selection of allele variants in functions

associated with c-di-GMP signalling networks. These signalling networks have a key role in several cellular functions, including attachment and motility, adhesion, biofilm formation, and exopolysaccharide production (Jenal *et al*., 2017), which can all impact success as an intracellular symbiont. More recently some diguanylate cyclases in *P. aeruginosa* have been proposed to act as molecular thermosensory devices, allowing bacteria to calibrate intracellular c-di-GMP cycling in response to temperature (Almblad *et al*., 2021). Temperature is likely to be an important influence for these inoculant bacteria, which evolved in tropical Brazilian soils and have now been introduced to temperate European soils. Further investigation is needed to determine the fitness effects of strains with altered/ loss of function diguanylate cyclase proteins.

Table 5A. Mutations found **at multiple field sites in *B. japonicum* SEMIA 5079**. Annotation column contains the position of amino acid codon changes, positions of the mutations within the gene for frameshift mutations or the position downstream (+) and upstream (-) for intergenic mutations. Syn = synonymous, Nonsyn = nonsynonymous. Average frequencies are reported for each field site (HF, NFF and TF) and for the overall total frequency as percentages ± standard error, calculated including zero values (*n* = 5).

Type	Position	Mutation	Annotation	Effect	Locus tag	RefSeq Protein Accession	Gene Description	HF Average Frequency	NFF Average Frequency	TF Average Frequency	Total Average Frequency
SNP	57885	T-G	L21R	Nonsyn	BJS_RS37475	WP_028151091.1	DUF1674 domain-containing protein	6 ± 1.8	5.3 ± 2.2	5.2 ± 1.6	5.5 ± 1
SNP	200977	A-C	(+76/-66)	Intergenic	BJS_RS38170 / BJS_RS38175	WP_038959153.1/ WP_039155347.1	*atpA* -F0F1 ATP synthase subunit alpha/ F0F1 ATP synthase subunit gamma	5.5 ± 2.9	1.4 ± 1.4	1.7 ± 1.7	2.7 ± 1.2
SNP	322175	A-T	(+61/-117)	Intergenic	BJS_RS38695 / BJS_RS38700	WP_039155430.1/WP _039155431.1	*hmgA* - homogentisate 1,2-dioxygenase/ *fahA* - fumarylacetoacetase	0 ± 0	2.6 ± 2.6	3.2 ± 2.9	2 ± 1.3
SNP	391165	T-G	C324G	Nonsyn	BJS_RS38975	WP_039155485.1	*phaA* - acetyl-CoA C-acetyltransferase	3.5 ± 3.1	9 ± 2.3	5 ± 3.1	6 ± 1.6
SNP	962221	C-A	S174S	Syn	BJS_RS41600	WP_039155973.1	ABC transporter permease	2.1 ± 1.9	7.2 ± 4.4	0 ± 0	3.2 ± 1.7
SNP	2004577	T-G	(+123/+49)	Intergenic	BJS_RS01755 / BJS_RS01760	WP_039145993.1/WP _039145996.1	*CheR* - protein-glutamate O-methyltransferase / glutathionylspermidine synthase family protein	14.6 ± 3.7	17.2 ± 1.2	9.1 ± 3.8	13.6 ± 1.9
SNP	2162908	A-C	C16G	Nonsyn	BJS_RS02495	WP_039146227.1	alpha/beta fold hydrolase	0 ± 0	3.1 ± 3.1	1.3 ± 1.1	1.5 ± 1.1
DEL	2288337	93 bp	(+70/-16)	Intergenic	BJS_RS03015 / BJS_RS03020	WP_014497457.1/ WP_039156558.1	TetR/AcrR family transcriptional regulator/ flavin reductase family protein	17 ± 4.4	17.5 ± 4.4	14.6 ± 6.3	16.3 ± 2.8
SNP	2790600	G-T	(-127/+12)	Intergenic	BJS_RS05555 / BJS_RS45670	WP_028156208.1/ N/A	hypothetical protein/ hypothetical protein	18.8 ± 4.7	25.2 ± 1	19.8 ± 5	21.3 ± 2.3
SNP	2920669	T-G	L270V	Nonsyn	BJS_RS06235	WP_039147320.1	LysR family transcriptional regulator	0 ± 0	4 ± 4	3 ± 3	2.3 ± 1.6
SNP	3253635	G-C	(+428/-23)	Intergenic	BJS_RS07970 / BJS_RS07975	WP_014494734.1/ N/A	hypothetical protein/ tRNA-Ser	1.6 ± 1.6	1.7 ± 1.7	2.1 ± 2.1	1.8 ± 1
SNP	3882078	C-T	(+39/-929)	Intergenic	BJS_RS10970 / BJS_RS10975	WP_014495284.1/ WP_051664586.1	hypothetical protein/ septal ring lytic transglycosylase RlpA family protein	2.8 ± 2.8	5.7 ± 3.5	0 ± 0	2.8 ± 1.5
SNP	3914397	G-T	A189E	Nonsyn	BJS_RS11110	WP_039148674.1	*fsrB* – siderophore utilisation protein - PepSY domain- coding protein	27 ± 13.6	53.1 ± 12	63.8 ± 18.8	48 ± 9.1
SNP	4161661	A-C	L35R	Nonsyn	BJS_RS12315	WP_014495522.1	*glpX* - fructose-bisphosphatase class II	21.9 ± 5.6	23.9 ± 6.1	21 ± 6.6	22.4 ± 3.2
SNP	5079372	C-A	(+172/-104)	Intergenic	BJS_RS16585 / BJS_RS16590	WP_148312052.1/ WP_232208317.1	helix-turn-helix transcriptional regulator/ glycosyltransferase family 1 protein	20.2 ± 14.2	40.6 ± 7	62.7 ± 18.9	41.1 ± 8.9
DEL	5084157	1 bp	(238/1881)	Frameshift	BJS_RS16605	WP_039150505.1	Polysaccharide chain length determinant N-terminal domain-containing protein	1.5 ± 1.5	8.5 ± 2.7	4.6 ± 3.1	4.9 ± 1.5
SNP	5122534	T-G	W198G	Nonsyn	BJS_RS16745	WP_039150552.1	amidohydrolase	5.5 ± 2.3	3.9 ± 2.4	3.7 ± 2.2	4.4 ± 1.3

Table 5A continued.

Type	Position	Mutation	Annotation	Effect	Locus tag	RefSeq Protein Accession	Gene Description	HF Average Frequency	NFF Average Frequency	TF Average Frequency	Total Average Frequency
SNP	5816354	T-G	(+88/-97)	Intergenic	BJS_RS19705 / BJS_RS19710	WP_014497036.1/ WP_232208603.1	XRE family transcriptional regulator/ murein L,D-transpeptidase	4.9 ± 3	4.5 ± 2.8	3.9 ± 2.6	4.4 ± 1.5
SNP	5975718	G-C	(-39/-245)	Intergenic	BJS_RS20385 / BJS_RS20390	WP_039151688.1/ WP_014497178.1	DNA-binding response regulator/ HlyD family type I secretion periplasmic adaptor subunit	2 ± 2	5.9 ± 3.7	0 ± 0	2.6 ± 1.5
SNP	6156967	C-G	(-204/+116)	Intergenic	BJS_RS21215 / BJS_RS21220	WP_232208560.1/ WP_232208543.1	DUF5801 domain-containing protein/ type I secretion system permease/ATPase	0 ± 0	3.9 ± 2.4	1.8 ± 1.8	1.9 ± 1
SNP	6217287	T-C	(-5/+171)	Intergenic	BJS_RS21505 / BJS_RS46195	WP_039151941.1/ WP_232208425.1	hypothetical protein/ hypothetical protein	37.3 ± 9.4	46.1 ± 0.9	47.8 ± 0.7	43.5 ± 3.3
DEL	6269332	13 bp	(-37/+156)	Intergenic	BJS_RS21810 / BJS_RS21815	WP_039152028.1/ WP_028158473.1	*adhP* - alcohol dehydrogenase / DUF4189 domain-containing protein	32.2 ± 13.2	22.4 ± 6.1	1.5 ± 1.5	18.7 ± 5.7
SNP	6274031	T-G	(-74/+67)	Intergenic	BJS_RS21835 / BJS_RS21840	WP_014494197.1/ WP_014494196.1	VWA domain-containing protein/ MoxR family ATPase	2.3 ± 2.3	3.7 ± 2.3	4.3 ± 2.2	3.4 ± 1.2
SNP	6541221	T-C	N363D	Nonsyn	BJS_RS23080	WP_039152306.1	hybrid sensor histidine kinase-response regulator – PAS-domain containing protein	25.6 ± 13.3	51.5 ± 12.3	61.4 ± 19	46.2 ± 9.1
SNP	7051858	T-G	A162A	Syn	BJS_RS25360	WP_039152849.1	alpha/beta hydrolase - AB hydrolase-1 domain-containing protein	6 ± 2.5	5.4 ± 2.2	6.3 ± 2	5.9 ± 1.2
SNP	7487481	T-A	(+162/+159)	Intergenic	BJS_RS27250 / BJS_RS27255	WP_039153072.1/ WP_014492925.1	glutamate-1-semialdehyde 2,1-aminomutase/ hypothetical protein	3.2 ± 3.2	3 ± 3	2.9 ± 2.6	3.1 ± 1.6
SNP	7714125	T-G	E336A	Nonsyn	BJS_RS28320	WP_014492712.1	ABC transporter substrate-binding protein	0 ± 0	2.4 ± 2.4	1.9 ± 1.9	1.4 ± 1
SNP	7937001	T-G	Q135P	Nonsyn	BJS_RS29430	WP_014492500.1	Transmembrane protein	2.7 ± 2.7	4.4 ± 2.7	2.3 ± 2	3.2 ± 1.4
SNP	8037217	C-A	K479N	Nonsyn	BJS_RS29905	WP_014492408.1	GGDEF-domain containing protein - Diguanylate cyclase-phosphodiesterase	0 ± 0	7 ± 7	0 ± 0	2.3 ± 2.3
SNP	8038025	A-G	L210P	Nonsyn	BJS_RS29905	WP_014492408.1	GGDEF-domain containing protein - Diguanylate cyclase-phosphodiesterase	14.3 ± 14.3	0 ± 0	2.3 ± 2.3	5.6 ± 4.8
SNP	8171744	A-C	S178R	Nonsyn	BJS_RS30550	WP_039153804.1	acyl-CoA dehydrogenase	1.7 ± 1.7	2.2 ± 2.2	1.3 ± 1.3	1.7 ± 1
SNP	8638244	A-G	I25T	Nonsyn	BJS_RS32800	WP_039154332.1	methylated-DNA--[protein]-cysteine S-methyltransferase	2.3 ± 2.3	6.8 ± 4.1	0 ± 0	3 ± 1.6
SNP	8846760	A-T	(+68/+120)	Intergenic	BJS_RS33850 / BJS_RS33855	WP_038957700.1/ WP_014491452.1	*PhnF* - phosphonate metabolism transcriptional regulator/ *PhnE* - phosphonate ABC transporter, permease protein	2.6 ± 2.6	0 ± 0	3.6 ± 3.6	2.1 ± 1.4
SNP	9248649	T-G	(-139/+43)	Intergenic	BJS_RS35665 / BJS_RS35670	WP_028170384.1/ WP_014491112.1	hypothetical protein/ OsmC family peroxiredoxin	2.4 ± 2.4	4.4 ± 2.7	4.2 ± 2.6	3.7 ± 1.4

Table 5B. Mutations found **at multiple field sites in *B. diazoefficiens* SEMIA 5080**. Annotation column contains the position of amino acid codon changes, positions of the mutations within the gene for frameshift mutations or the position downstream (+) and upstream (-) for intergenic mutations. Syn = synonymous, Nonsyn = nonsynonymous. . Average frequencies are reported for each field site (HF, NFF and TF) and for the overall total frequency as percentages ± standard error, calculated including zero values (*n* = 5).

Type	Position	Mutation	Annotation	Effect	Locus tag	RefSeq Protein Accession	Gene Description	HF Average Frequency	NFF Average Frequency	TF Average Frequency	Total Average Frequency
SNP	81554	G-A	V691M	Nonsyn	BJA5080_RS27990	WP_028175099.1	amino acid ABC transporter substrate-binding protein	68.8 ± 20.7	24.3 ± 12.7	68.1 ± 21.1	53.7 ± 11.2
SNP	89927	T-G	H15P	Nonsyn	BJA5080_RS28035	WP_011082921.1	ABC transporter permease	8.2 ± 2.6	4.5 ± 2.4	7.7 ± 2.4	6.8 ± 1.3
SNP	444106	T-G	A25A	Syn	BJA5080_RS29620	WP_028174835.1	diguanylate phosphodiesterase	3.4 ± 3	3.1 ± 2.8	9.6 ± 2.9	5.4 ± 1.7
SNP	1277500	G-T	(-529/-19)	Intergenic	BJA5080_RS33375 / BJA5080_RS33380	WP_028172787.1/ N/A	hypothetical protein/ tRNA-Gly	2 ± 1.8	25 ± 22.4	0 ± 0	9 ± 7.4
SNP	2151522	C-A	L183F	Nonsyn	BJA5080_RS27350	N/A	hypothetical protein	67.3 ± 20.3	25.7 ± 13.3	68.1 ± 21.1	53.7 ± 11.1
SNP	2448480	T-C	L271P	Nonsyn	BJA5080_RS26170	WP_028175163.1	AraC family transcriptional regulator	27.9 ± 21.5	37 ± 17.6	0 ± 0	21.6 ± 9.4
SNP	3182779	C-T	G179G	Syn	BJA5080_RS22895	WP_039185714.1	ABC transporter ATP-binding protein	26.8 ± 21.9	31.6 ± 21.1	0 ± 0	19.5 ± 9.9
DEL	5669631	1 bp	(520/621)	Frameshift	BJA5080_RS10835	WP_028171054.1	hypothetical protein	25 ± 22.4	14.3 ± 8	0 ± 0	13.1 ± 7.7
DEL	7761910	7 bp	(708-714/963)	Frameshift	BJA5080_RS01650	WP_028174247.1	2-pyrone-4,6-dicarboxylate hydrolase -	1.7 ± 1.5	23.5 ± 21.1	0 ± 0	8.4 ± 7
SNP	8149795	A-T	(+62/+123)	Intergenic	BJA5080_RS37205 / BJA5080_RS37210	WP_028172968.1/ WP_011089945.1	type 1 glutamine amidotransferase/ DUF1348 domain-containing protein – nuclear transport factor 2 family protein	2.9 ± 2.5	0 ± 0	12.8 ± 3.8	5.2 ± 2
SNP	8223426	A-C	S178R	Nonsyn	BJA5080_RS37535	WP_028172925.1	acyl-CoA dehydrogenase	2.3 ± 2.1	0 ± 0	7.4 ± 2.2	3.3 ± 1.2
SNP	8391008	A-C	(-106/+118)	Intergenic	BJA5080_RS38305 / BJA5080_RS38310	WP_028174703.1/ WP_028174702.1	MFS transporter/ *glmM* - phosphoglucosamine mutase	2.6 ± 2.3	0 ± 0	3.9 ± 2	2.2 ± 1
SNP	8549210	T-G	A119A	Syn	BJA5080_RS39130	WP_028173925.1	UDP-hexose transferase	3.7 ± 1.9	0 ± 0	7.3 ± 2.2	3.7 ± 1.2
SNP	8974342	A-C	F111V	Nonsyn	BJA5080_RS40985	WP_028175978.1	*truA* - tRNA pseudouridine(38-40) synthase	4 ± 2.1	2.6 ± 2.3	6.7 ± 2	4.4 ± 1.2
SNP	9045509	A-C	L297R	Nonsyn	BJA5080_RS41295	WP_028175936.1	Potassium-proton antiporter- Putative Cell volume regulation protein A	7 ± 3.6	0 ± 0	11.2 ± 3.4	6.1 ± 2

Polymorphisms unique to field sites

Mutations unique to field sites (Table 6A and 6B), often appear at much lower average frequencies than mutations found across field sites, because they are largely unique to plant replicates. In *B. japonicum* 12.5% of site specific mutations are at 100% frequency and in *B. diazoefficiens*, 30% of mutations are at 100% frequency within one replicate nodule community. This could suggest selection of novel variants locally within or around plant replicates. Alternatively, as the communities were assessed after their first introduction to these field sites, effectively, after a short period in the introduced soil microbiome and one population bottleneck (plant selection for nodulation), the frequency and occurrence of these mutations could suggest genetic drift in these contained nodule populations.

Notably, there appears to be less functional overlap between the genes possessing mutations, however some mutations may still impact symbiotic traits. For example, In *B. japonicum*, there is a tolerated missense polymorphism in a glutathione S-transferase family protein (BJS_RS12490) that occurs in one replicate (3.28% average frequency) at site HF (Table 6A). Glutathione S-transferases are involved in the protection of the N_2-fixing process against reactive oxygen species (ROS) resulting from active nodule metabolism (Bianucci *et al*., 2017), which can protect nodules from senescence (Ohkama-Ohtsu *et al*., 2016). In *B. japonicum*, there is further evidence of mutations occuring in ABC transporter proteins that are unique to replicates within sites, one synonymous polymorphism at site TF (BJS_RS33110), one synonymous polymorphism (BJS_RS25510) and a tolerated missense polymorphism (BJS_RS25045) at site NFF (Table 6A). Additionally, In B. japonicum, there is a tolerated missense polymorphism in dicarboxylate--CoA ligase *PimA* (BJS_RS42415), which is only present in site TF occurring in one plant replicate to 34% frequency (Table 6A). Dicarboxylates, such as malate, succinate and fumarate are the primary source of carbon provided to rhizobia symbionts by the plant (Udvardi and Poole, 2013) and thus their

incorporation into, and metabolism via the TCA cycle is important for bacteroid fitness and essential for N_2 fixation.

For *B. diazoefficiens,* the largest number of mutations were found at site TF (Table 6B). There is an intergenic SNP 28 bp upstream of TonB- dependent receptor (BJA5080_RS36530) and 217 bp upstream of hemin uptake protein HemP (BJA5080_RS36535). The TonB receptor is of the haemoglobin/transferrin/lactoferrin family, therefore important in heme/ haemoglobin uptake, an essential element in maintaining low O^2 conditions for nitrogenase ability (Lim, 2010). Within the same plant replicate there is a tolerated missense mutation in a FecR domain- containing protein (BJA5080_RS19185) involved in ferric- citrate uptake (Enz *et al*., 2000). In *B. diazoefficiens,* There are also two frameshift mutations that will likely impact protein function. At site TF there is a 1bp deletion in a CoA-binding protein (BJA5080_RS07820), essential in metabolism, in one replicate at 100%. At site HF there is a 2 bp deletion in a FAD- binding protein, involved in oxidoreductase activity, present in one replicate at 100% frequency.

Table 6A. Mutations **unique to field sites in *B. japonicum* SEMIA 5079**. Annotation column contains the position of amino acid codon changes, positions of the mutations within the gene for frameshift mutations or the position downstream (+) and upstream (-) for intergenic mutations. Syn = synonymous, Nonsyn = nonsynonymous. Average frequencies are reported for each field site (HF, NFF and TF) and for the overall total frequency as percentages ± standard error, calculated including zero values (*n* = 5).

Type	Position	Mutation	Annotation	Effect	Locus Tag	RefSeq Protein Accession	Gene Description	HF Average Frequency	NFF Average Frequency	TF Average Frequency	Total Average Frequency
SNP	388730	C-A	E153D	Nonsyn	BJS_RS38965	WP_039155483.1	class I SAM-dependent methyltransferase	2.75 ± 2.46	0 ± 0	0 ± 0	0.79 ± 0.76
SNP	2014674	G-A	(-89/+207)	Intergenic	BJS_RS01810 / BJS_RS01815	WP_014497685.1/ WP_039146019.1	hypothetical protein/ *FlhA*- flagellar biosynthesis protein	2.2 ± 2.2	0 ± 0	0 ± 0	0.73 ± 0.73
SNP	4198164	C-T	G104S	Nonsyn	BJS_RS12490	WP_039157012.1	glutathione S-transferase family protein	3.28 ± 3.28	0 ± 0	0 ± 0	1.09 ± 1.09
SNP	4580341	G-A	I190I	Syn	BJS_RS14380	WP_014495948.1	methylmalonate-semialdehyde dehydrogenase (CoA acylating)	4.06 ± 4.06	0 ± 0	0 ± 0	1.35 ± 1.35
DEL	4812195	57 bp	(-73/+426)	Intergenic	BJS_RS15465 / BJS_RS15470	WP_038932082.1/ WP_039150093.1	*TerB* Tellurite resistance protein / nuclear transport factor 2 family protein	2.24 ± 2.24	0 ± 0	0 ± 0	0.75 ± 0.75
SNP	5957069	G-T	(-39/-245)	Intergenic	BJS_RS20310 / BJS_RS20315	WP_039151671.1/ WP_039151672.1	hypothetical protein/hypothetical protein	6.5 ± 4.47	0 ± 0	0 ± 0	2.17 ± 1.6
SNP	6037726	A-C	(-5/+171)	Intergenic	BJS_RS20590 / BJS_RS20595	WP_039151734.1/ WP_039151736.1	hypothetical protein - HTH marR-type domain-containing protein/ hypothetical protein	4.5 ± 4.5	0 ± 0	0 ± 0	1.5 ± 1.5
SNP	7156283	T-C	(-153/+541)	Intergenic	BJS_RS25775 / BJS_RS25780	WP_148312209.1/ WP_014493473.1	hypothetical protein- Rap1a-Tai family immunity protein/ hypothetical protein	4 ± 4	0 ± 0	0 ± 0	1.33 ± 1.33
SNP	7687262	T-A	(+50/+154)	Intergenic	BJS_RS28185 / BJS_RS28190	WP_014492739.1/ WP_039153290.1	IclR family transcriptional regulator/ NAD(P)-dependent SDR family oxidoreductase	3.52 ± 3.52	0 ± 0	0 ± 0	1.17 ± 1.17
SNP	8605089	G-T	P126P	Syn	BJS_RS32660	WP_038935562.1	*fdh3b* - formate dehydrogenase FDH3 subunit beta	3.62 ± 3.62	0 ± 0	0 ± 0	1.21 ± 1.21
SNP	1130311	A-C	Q544P	Nonsyn	BJS_RS42415	WP_039156166.1	*PimA* - dicarboxylate--CoA ligase	0 ± 0	0 ± 0	6.88 ± 6.88	2.46 ± 2.37
INS	2200437	+C	(54/423)	Frameshift	BJS_RS02660	WP_039146280.1	hypothetical protein	0 ± 0	0 ± 0	20 ± 20	6.67 ± 6.67

Table 6A continued.

Type	Position	Mutation	Annotation	Effect	Locus Tag	RefSeq Protein Accession	Gene Description	HF Average Frequency	NFF Average Frequency	TF Average Frequency	Total Average Frequency
INS	3748687	+G	(322/938)	Frameshift	BJS_RS10325	N/A	pseudogene - hypothetical protein	0 ± 0	0 ± 0	20 ± 20	6.67 ± 6.67
SNP	4325455	T-G	(+58/-265)	Intergenic	BJS_RS13095 / BJS_RS13100	WP_039149305.1/ WP_014495683.1	hypothetical protein/ PilZ domain-containing protein	0 ± 0	0 ± 0	4.38 ± 2.7	1.46 ± 1
SNP	4589445	T-G	S32R	Nonsyn	BJS_RS14420	WP_039149775.1	cobalt transporter	0 ± 0	0 ± 0	2.57 ± 2.3	0.74 ± 0.71
SNP	5732981	G-A	S612S	Syn	BJS_RS19350	WP_014496962.1	MexW/MexI family multidrug efflux RND transporter permease subunit	0 ± 0	0 ± 0	3.22 ± 3.22	1.07 ± 1.07
SNP	8702924	G-A	E24E	Syn	BJS_RS33110	WP_039154394.1	branched-chain amino acid ABC transporter substrate-binding protein	0 ± 0	0 ± 0	20 ± 20	6.67 ± 6.67
SNP	2501900	C-T	pseudogene	Syn	BJS_RS03985	WP_051664500.1	pseudogene - MFS transporter	0 ± 0	7.34 ± 7.34	0 ± 0	2.45 ± 2.45
SNP	2954315	G-T	E214*	nonsense	BJS_RS06405	WP_039156827.1	porin family protein	0 ± 0	2.66 ± 2.66	0 ± 0	0.89 ± 0.89
SNP	4971531	T-C	G388G	Syn	BJS_RS16145	WP_039150352.1	Oxidoreductase - Rieske 2Fe-2S domain-containing protein	0 ± 0	2.8 ± 2.8	0 ± 0	0.93 ± 0.93
SNP	6968123	T-C	K3R	Nonsyn	BJS_RS25045	WP_039157633.1	ABC transporter substrate-binding protein	0 ± 0	2.9 ± 2.9	0 ± 0	0.97 ± 0.97
SNP	7085237	G-C	P238P	Syn	BJS_RS25510	WP_014493525.1	ABC transporter permease	0 ± 0	2.96 ± 2.96	0 ± 0	0.99 ± 0.99
SNP	7190630	A-C	V130G	Nonsyn	BJS_RS25905	WP_039152909.1	*egtD* - L-histidine N(alpha)-methyltransferase	0 ± 0	2.56 ± 2.56	0 ± 0	0.85 ± 0.85
SNP	8332820	G-A	G104G	Syn	BJS_RS31345	WP_014491991.1	*ribH* - riboflavin synthase subunit beta - 6,7-dimethyl-8-ribityllumazine synthase	0 ± 0	2.84 ± 2.84	0 ± 0	0.95 ± 0.95

Table 6B. Mutations **unique to field sites in *B. diazoefficiens* SEMIA 5080**. Annotation column contains the position of amino acid codon changes, positions of the mutations within the gene for frameshift mutations or the position downstream (+) and upstream (-) for intergenic mutations. Syn = synonymous, Nonsyn = nonsynonymous. Average frequencies are reported for each field site (HF, NFF and TF) and for the overall total frequency as percentages ± standard error, calculated including zero values (*n* = 5).

Type	Position	Mutation	Annotation	Effect	Locus Tag	Refseq Protein Accession	Gene Description	HF Average Frequency	NFF Average Frequency	TF Average Frequency	Total Average Frequency
SNP	1600779	C-A	P885P	Syn	BJA5080_RS34990	WP_028173519.1	*metH* - methionine synthase	21.7 ± 19.4	0 ± 0	0 ± 0	7.2 ± 6.5
SNP	5132628	G-A	R102H	Nonsyn	BJA5080_RS13490	WP_028174040.1	MBL fold metallo-hydrolase	25 ± 22.4	0 ± 0	0 ± 0	8.3 ± 7.5
SNP	5954412	C-G	E188D	Nonsyn	BJA5080_RS09475	WP_028170848.1	hypothetical protein	5.4 ± 4.9	0 ± 0	0 ± 0	1.8 ± 1.6
DEL	6102611	2 bp	(695-696/1704)	Frameshift	BJA5080_RS08880	WP_028170752.1	FAD-dependent oxidoreductase	25 ± 22.4	0 ± 0	0 ± 0	8.3 ± 7.5
DEL	2828093	79 bp	(+55/-59)	Intergenic	BJA5080_RS24420 / BJA5080_RS24415	WP_028176244.1/ WP_028176243.1	*murC* - UDP-N-acetylmuramate--L-alanine ligase/ *murB* - UDP-N-acetylenolpyruvoylglucosamine reductase	0 ± 0	0 ± 0	5.9 ± 3.1	2 ± 1.2
SNP	2911444	A-T	Q217L	Nonsyn	BJA5080_RS24080	WP_028176188.1	DUF2336 domain-containing protein	0 ± 0	0 ± 0	17.7 ± 15.9	5.9 ± 5.3
SNP	3957908	A-C	C40G	Nonsyn	BJA5080_RS19185	WP_028175444.1	fecR domain-containing membrane protein	0 ± 0	0 ± 0	6.1 ± 5.4	2 ± 1.8
SNP	4195855	A-T	(+63/-145)	Intergenic	BJA5080_RS18110 / BJA5080_RS18105	WP_028171358.1/ WP_028171359.1	CoA transferase/3-keto-5-aminohexanoate cleavage protein	0 ± 0	0 ± 0	5.9 ± 5.3	2 ± 1.8
SNP	5735945	A-C	(-241/-167)	Intergenic	BJA5080_RS10465 / BJA5080_RS10460	WP_011086749.1/ WP_011086748.1	*guaB* - IMP dehydrogenase/MFS transporter	0 ± 0	0 ± 0	3.1 ± 2.8	1 ± 0.9
SNP	6120527	G-C	P299R	Nonsyn	BJA5080_RS08805	WP_231166691.1	O-antigen ligase domain-containing protein	0 ± 0	0 ± 0	6 ± 5.4	2 ± 1.8
SNP	6169277	G-C	T70T	Syn	BJA5080_RS08585	WP_231166687.1	DUF4384 domain-containing protein	0 ± 0	0 ± 0	25 ± 22.4	8.3 ± 7.5
DEL	6339511	1 bp	(1802/2133)	Frameshift	BJA5080_RS07820	WP_028171981.1	CoA-binding protein - acetate--CoA ligase family protein	0 ± 0	0 ± 0	25 ± 22.4	8.3 ± 7.5
SNP	7402058	G-A	E174K	Nonsyn	BJA5080_RS03250	WP_028175370.1	diguanylate cyclase - HAMP domain-containing protein	0 ± 0	0 ± 0	25 ± 22.4	8.3 ± 7.5
SNP	8009880	C-G	(-28/-217)	Intergenic	BJA5080_RS36530 / BJA5080_RS36535	WP_028173051.1/ N/A	TonB-dependent receptor/hemin uptake protein HemP	0 ± 0	0 ± 0	3.2 ± 2.9	1.1 ± 1
SNP	4473371	G-A	K285K	Syn	BJA5080_RS16630	WP_011087918.1	alpha/beta hydrolase - AB hydrolase-1 domain-containing protein	0 ± 0	17.6 ± 15.8	0 ± 0	5.9 ± 5.3
SNP	6189647	C-A	T373K	Nonsyn	BJA5080_RS08515	WP_028170698.1	type VI secretion system ImpA family N-terminal domain-containing protein	0 ± 0	8.9 ± 8	0 ± 0	3 ± 2.7
DEL	7685032	10 bp	(283-292/354)	Frameshift	BJA5080_RS01995	WP_028174305.1	hypothetical protein	0 ± 0	15.8 ± 14.1	0 ± 0	5.2 ± 4.7

Absence of mutations in symbiosis elements

There is a notable absence of polymorphisms within the symbiosis islands of the inoculant genomes (Figure 5). The inoculant strains possess a tRNA-valine bipartite ICE, which splits into two regions (A and B) when integrated into the genome (see Figure 5; Weisberg *et al.,* 2022a). A bootstrapping simulation was performed to generate random positions of mutations (58 for *B. japonicum* and 32 for *B. diazoefficiens*) in the inoculant genomes for 1000 simulations. In 98.5% (*B. japonicum*) and 93.4% (*B. diazoefficiens*) of simulations, mutations were present within the ICE^{sym} A or B elements, suggesting that by chance we would expect mutations to occur within these regions. There is, however, a tolerated missense polymorphism that occurred within the *B. diazoefficiens* larger ICE^{sym} A element, in a hypothetical gene (BJA5080_RS27350; Table 5B). This polymorphism is at quite high average frequency in sites HF (67.3%) and site TF (68.1%) but lower at site NFF (25.7%). The hypothetical protein has a 100% identity match with a *Bradyrhizobium* multispecies DUF5716 family protein (NCBI protein accession no: WP_011084477.1). Unfortunately, no known function is associated with this predicted protein, however the polymorphism occurs within a JetA domain (Interpro). JetA is part of the Wadjet anti-plasmid defence system (JetABCD) which protects bacterial hosts from plasmid transformation (Deep *et al.*, 2022; Liu, Roisné-Hamelin and Gruber, 2023) and therefore may play a role in competition with invading MGEs. This highlights that successful variants present in the nodule communities have highly conserved symbiosis elements. Comparative genomics analysis has revealed that symbiosis genes are often under strong purifying selection (Epstein and Tiffin, 2021), which maintains their function as symbionts.

5.5 Conclusions

Inoculants dominate nodule occupancy, but %Ndfa across sites is variable

The inoculant strains dominated the nodule populations as expected, since they were the only compatible rhizobial symbionts in the soil microbiome. However, there were also other *Bradyrhizobium* species detected at extremely low abundance in the nodule microbiomes. This may suggest that some members of the native population can enter nodules alongside compatible symbionts (Zgadzaj *et al*., 2015), or potentially they have acquired the symbiosis genes via HGT. Research in *Mesorhizobium* species (which also carry ICE^{syms}) has shown that native, nonsymbiotic genospecies are capable of acquiring ICE^{syms} and becoming rhizobial symbionts with varying effectiveness (Colombi *et al*., 2023, 2021; Haskett *et al*., 2016; Hill *et al*., 2021; Sullivan *et al*., 1995). Although HGT of symbiosis genes between ICE^{syms} appears common (Weisberg *et al*., 2022a), there is less evidence of whole ICE^{sym} mobilisation into nonsymbiotic *Bradyrhizobium* (Barcellos *et al*., 2007; Minamisawa *et al*., 2002). Interestingly, the mobility potential of the inoculants SEMIA 5080 and SEMIA 5079 bipartite tRNA- Valine ICE^{syms} has been questioned, due to degradation of the *att* sites that are required for ICE^{sym} integration and excision in these strains (private correspondence with A. Weisberg). There was a diverse native *Bradyrhizobium* community in the soil microbiome, including known symbiotic and non-symbiotic genospecies, however native gorse and broom failed to produce N_2-fixing nodules in these soils, suggesting their symbiotic rhizobial partners were absent. This may be because agricultural soil is not a natural environment for gorse and broom, which thrive in more acidic, nutrient depleted soils (Leary *et al*., 2006) and could suggest that HGT between native gorse and broom *Bradyrhizobium* symbionts and soya *Bradyrhizobium* symbionts may be unlikely due to the little overlap in their soil environments.

The high dominance of the inoculant strains in the nodules suggests they were the dominant N_2 fixers and despite the same soybean cultivar used, %Ndfa in plant samples varied across field sites. The %Ndfa values were lower than previously reported for temperate soybean cultivars grown in Scotland, which were relatively high (65 - 76%; Maluk *et al.*, 2023), but fell within the range for average European grown soybeans (44 ± 13 %;

Peoples *et al.*, 2021). BNF can be influenced by the surrounding soil and climatic environment, for example increased soil P content and effective rainfall up until flowering are predictors of %Ndfa increases (Collino *et al*., 2015). Significant correlations were found between soil P and plant N content in this study, however a negative correlation was found between soil moisture content and %Ndfa, potentially as SMC was suboptimal during the early pod fill growth stage, due to the wet early growth season of 2021. Further research investigating the factors involved in optimising soybean BNF in the UK could improve the uptake of this underutilised crop (DEFRA, 2022) particularly as the changing climate increases the range where temperate varieties can be grown (Coleman *et al*., 2021).

Variant pools within nodule environments

This study investigated the potential for inoculant evolution during the first season of soybean cropping. The nodule communities provided a snapshot in evolutionary time for the introduced inoculant strains, allowing identification of genetic variants *in situ*. There was little evidence of polymorphisms within the symbiosis elements, which makes sense in the light of host selection, as these were the only compatible symbiosis genes in the environment. After a prolonged period of adaptation in the soil microbiome, ICE^{sym} variants may arise, as observed in other introduced regions of the world (Barcellos *et al*., 2007; Batista *et al*., 2007; Iturralde *et al*., 2019; Satya Prakash and Annapurna, 2006). Additionally, only the pool of successful symbionts were assessed, isolation and sequencing strains from rhizosphere communities in future research may highlight symbiosis element variants that didn't make it into nodule communities. It is interesting that the only ICE^{sym} polymorphism occurred within a *B. diazoefficiens* protein that may play a role in anti-plasmid defence. Further investigation into this protein is needed to identify whether it is a component of the WadJet system (Deep *et al*., 2022), and may highlight how the resident MGE can carry mechanisms to compete with other incoming MGEs. Direct conflicts between MGEs in bacterial hosts can occur, which can have effects on bacterial fitness (Hall *et al*., 2021). If MGEs can reduce

competition from other MGEs it may provide an advantage, for example in a *Mesorhizobium* isolate, a type I-C CRISPR-Cas system was found on the largest ICE^{sym} (AA22) known to date, which carried targets similar to putative phage and plasmid sequences found in other soil bacteria (Colombi *et al*., 2021).

Mutations outside of the ICE^{sym} elements can still affect the capability of strains as symbionts, as many genes are required for rhizosphere colonisation through to N_2 - fixing bacteroids and contribute to the competitiveness of symbionts (Burghardt and diCenzo, 2023; Mendoza-Suárez *et al*., 2021; Poole *et al*., 2018; Wheatley *et al*., 2020). There were more mutations present in *B. japonicum* compared to *B. diazoefficiens*, which was also found for these inoculant strains, when only assessing the genetic variation of the symbiosis elements of the parental and closely related strains to SEMIA 5080 and SEMIA 5079 (Bender *et al*., 2022). Additionally, some of the closely related strains had no polymorphisms in their symbiosis regions, but still varied in competitiveness and BNF efficiency, highlighting that symbiotic phenotypes were due to differences outside of the ICE^{sym} regions (Bender *et al*., 2022).

Notably, there was polymorphic diversity in bacterial genes involved in transport and metabolic processes, particularly C metabolism across both inoculant species, and N metabolism in *B. japonicum*. N metabolism has been suggested to be upregulated in soybean bacteroids, but C cycling remains similar between free-living and bacteroid states (Sarma and Emerich, 2006). Amino acid uptake and cycling has been shown to be important in *R. leguminosarum* indeterminate bacteroids to ensure ammonium, the primary N export, is not assimilated by the bacteria (Lodwig *et al*., 2003; Prell and Poole, 2006), however alanine and aspartate have also been shown to be transported to the plant from determinate soybean bacteroids (Waters *et al*., 1998; Whitehead *et al*., 1998). Polymorphisms in C metabolism and transporter proteins could reflect the need to adapt to variations in photosynthates provided by a new plant host (a temperate soybean cultivar) in this new

environment (UK agricultural soil). Research from rhizobia experimental evolution studies has recently found that after successive nodulation cycles, rhizobia evolved to enhance plant host benefits, not by improved N_2 fixation which was expected, but by reducing the cost of the symbiosis via changes in metabolic requirements (Quides *et al*., 2021). Rapid adaptation of rhizobia to local host genotypes has been observed in *S. meliloti*, where adaptation was mostly limited to the symbiosis plasmids, however variants also arose in other genetic regions, with some functional overlap of mutated genes found in this study (*e.g*. variants in an ATP synthase subunit gene, ABC transporter ATP- binding protein gene and FAD-binding oxidoreductases; supplementary material Batstone *et al.*, 2020).

Surprisingly, there was a high amount of polymorphic genetic diversity in the communities, in which 47% - 57% was present across all three field sites, suggesting a large amount of diversification has happened prior to introduction. This could imply that the inoculant production process may be introducing genetic variation in inoculant populations, from which plant hosts select. In the nutrient dense production vat there may be no cost or advantage to these sporadic mutations. However, once introduced to agricultural environments, variants may be subjected to different selection pressures, where acquired mutations may prove detrimental or advantageous to bacterial fitness. Additionally, 53% (*B diazoefficiens*) and 43% (*B. japonicum*) of mutations were unique to field sites, and largely unique to plant replicates, suggesting they may have arisen post introduction, during the ~4 months where inoculant populations experienced various life stages, on the seed coat, in the rhizosphere, in infection threads and within nodule environments. Each of these stages has been shown to require specific genes (Wheatley *et al*., 2020) and specific adaptations (Ledermann *et al*., 2021).

Strong selection imposed by host plants can further shape the evolution of these symbiont populations (Doin de Moura *et al*., 2023) and effective host sanctions can reduce less efficient N fixers in nodule populations (Kiers *et al*., 2003; Westhoek *et al*., 2021, 2017).

Experimental evolution of a plant pathogen into a rhizobial symbiont has highlighted that most of the adaptive mutations that occurred during this process arose *ex planta,* in the rhizosphere (Doin de Moura *et al*., 2023). Interestingly, adaptive sweeps were defined by cohorts of beneficial mutations, aided by hyper mutagenesis genes that are often carried on rhizobial plasmids (Remigi *et al*., 2014). Although the presence of hyper-mutagenesis cassettes on ICE^{syms} is unknown (Remigi *et al*., 2016), these regions do contain a large amount of insertion sequence elements (IS; Barros-Carvalho *et al.*, 2019), for which a similar phenomenon has been observed where IS-mediated mutations often occur during rhizobial saprophytic growth and from which plant hosts select (Arashida *et al*., 2022).

The potential evolutionary effect of inoculant production may confer some benefit, for example, the introduction of a diverse intraspecific inoculant to a new environment may increase the chances of beneficial bacterial genotype x host genotype x environment (G x G x E) interactions. Subsequent rounds of host plant growth, without further inoculation in these regions may naturally select the most beneficial symbionts from previously successful symbiont pools that may have adapted to local edaphic conditions. Future research should focus on assessing the inputs to and output from inoculant production processes, at the multiple stages involved, past research has largely focused on the issue of contamination by culturable methods (Catroux, Hartmann and Revellin, 2001), but with advances in sequencing and bioinformatics approaches, the genetic composition of inoculant products can be investigated. Evolution of inoculant strains via local adaptation over time (Hungria *et al*., 1996; Santos *et al*., 1999) or HGT of symbiosis genes (Barcellos *et al*., 2007; Batista *et al*., 2007; Hill *et al*., 2021; Nandasena *et al*., 2007, 2006; Sullivan *et al*., 1995) has been documented, but here a potential role for the production process in inoculant evolution was found.

5.6 Acknowledgements

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Table S1. Soybean field sample sites metadata.

Field Site	Sample coordinates	Cropping History	Soil type (UKSO)	pH	P (ppm)	K (ppm)	Mg (ppm)	NO3-N (ppm)	NH4+N (ppm)	No. Nodules pooled for sequencing
HF	52°53'19.9"N 0°19'00.6"W	2020: Grain Maize 2019: Winter Wheat 2018: Peas 2017: Winter Wheat 2016: Winter O.S.R.	Sandy Loam	7.67 ± 0.105	38 ± 3.5	154.2 ± 15.3	43.3 ± 2.73	-0.442 ± 0.450	1.60 ± 0.372	Rep 1: 11 Rep 2: 50 Rep 3: 6 Rep 4: 38 Rep 5: 3
NFF	52°22'58.7"N 0°02'07.7"E	2020: Onions 2019: Wheat Prior to 2019: 'reclaimed by nature' (weeds)	Sand to Sandy Loam	7.55 ± 0.056	13.8 ± 2.2	253.3 ± 33.1	99.3 ± 5.54	7.81 ± 2.03	2.63 ± 0.655	Rep 1: 43 Rep 2: 50 Rep 3: 16 Rep 4: 20 Rep 5: 6
SF	52°57'02.4"N 0°06'54.9"W	2020: Wheat 2019: Wheat 2018: Wheat 2017: Cabbage	Clayey Loam to Silty Loam	7.22 ± 0.404	19.5 ± 1.9	261.2 ± 12.7	330 ± 21.3	39.5 ± 8.03	3.53 ± 1.21	N/A Not enough
GF	52°24'01.3"N 0°38'32.9"W	2021: Winter cover crop Dicon radish, vetch, black oats 2020: wheat 2019: Oil seed rape 2018: Wheat 2017: Beans 2016: Grass	Loam to Clayey Loam	6.78 ± 0.298	26.5 ± 3.9	404 ± 31	201.3 ± 16.1	15.4 ± 5.71	2.48 ± 0.496	N/A Not enough
TF	52°37'05.1"N 0°00'04.7"E	2020: Maize 2019: Maize 2018: Wheat 2017: Peas	Clayey Loam to Silty Loam	8.1 ± 0.044	11.2 ± 1.1	342 ± 40.6	167 ± 17.8	0.461 ± 1.02	1.96 ± 0.423	Rep 1: 13 Rep 2: 50 Rep 3: 3 Rep 4: 3 Rep 5: 1

Table S2. Primers and PCR Protocols used in this study.

Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	PCR programme	Reference
16S V4 (Amplicon sequencing primers with tags for secondary nested PCR highlighted in red)	ACACTCTTTCCCTACACGACGCTCT TCCGATCTNNNNNGTGCCAGCMGC CGCGGTAA	GTGACTGGAGTTCAGACGTGTGCTC TTCCGATCTGGACTACHVGGGTWTC TAAT	95°C for 5 min 25 x (94°C for 10s, 70.6°C for 30s, 72°C for 30s), 72°C for 5 min	Caporaso *et al*., 2011
rpoB (Amplicon sequencing primers with tags for secondary nested PCR highlighted in red)	ACACTCTTTCCCTACACGACGCTCT TCCGATCTGGYCGCGTSAARATGA ACATGCG	GTGACTGGAGTTCAGACGTGTGCTC TTCCGATCTGCRTTGATSAGRTCYT GYGGCATSAC	98 °C for 30s 30 x (98°C for 10s, 72 °C for 10s) 72 °Cfor 5 min	Chapter 2
BOXAIR	CTACGGCAAGGCGACGCTGACG	N/A	94°C for 30s 35 x (94°C for 10s, 50°C for 30s, 72°C for 30s) 72°C for 10min	Versalovic *et al*., 1996
rpoB Bj/Bd multiplex	Bjdrpob1_F (1uL @ 10uM): GAAGGCGCTGCGSCTGT Bdrpob2_F (0.5uL @ 10uM): GATGGTCGACGAACCCCAG	Bjdrpob1_R (1uL @ 10uM): TGCTCGTTGAGGGCCTTCAT	94 for 5 min, (94 for 10s, 65 for 30s, 72 for 30s) x 30, 72 for 5 min	Chapter 3
BnodZ2	TCGTCCTCGAGCAGGTTTCGGTTAA	CGAAGCCATAAGCGCTTGCGAGT	95°C for 5 min 30 x (95°C for 10s, 69°C for 30s, 72°C for 30s) 72°C for 5 min	Chapter 2

Table S3. Broom Rhizobia *rpoB* fragments isolated from SF, GF, HF field site soils.

Broom Isolate	Farm Site - Soil	Top Blast Hit	Fasta Sequence
SFB1	SF - Cereals Soil	100% identity to: • Rhizobium leguminosarum bv. trifolii TA1 • Rhizobium leguminosarum bv. trifolii strain 22B • Rhizobium leguminosarum bv. trifolii strain 31B • Rhizobium ruizarguesonis strain TA1 • Rhizobium leguminosarum strain CCGM4 • Rhizobium ruizarguesonis strain RCAM 1026 • Rhizobium leguminosarum bv. viciae strain RCAM0626 • Rhizobium leguminosarum bv. viciae strain RCAM2802 • Rhizobium leguminosarum strain CCBAU 01237	>SFB1 GACATCCTGGCCGTGGTCAGGATGCTGGTCGAACTGCGCGACGGCAAGGGCGAGATCG ACGACATCGACAACCTCGGCAACCGCCGCGTCCGTTCGGTCGGCGAGCTGATGGAAAAC CAGTACCGTCTCGGCCTGCTGCGCATGGAGCGCGCGATCAAGGAACGCATGTCCTCGAT CGAGATCGACACGGT
GFB1	GF - Cereals Soil	99.47% identity to: • Rhizobium leguminosarum bv. trifolii TA1 • Rhizobium leguminosarum bv. trifolii strain 22B • Rhizobium leguminosarum bv. trifolii strain 31B • Rhizobium leguminosarum strain A1 • Rhizobium ruizarguesonis strain TA1 • Rhizobium leguminosarum strain CCGM4 • Rhizobium ruizarguesonis strain RCAM 1026 • Rhizobium ruizarguesonis strain NZLR24 • Rhizobium leguminosarum bv. viciae strain RCAM0626 • Rhizobium leguminosarum bv. viciae strain RCAM2802 • Rhizobium leguminosarum strain CCBAU 01237	>GFB1 GACATCCTGGCCGTGGTCAGGATGCTGGTCGAACTGCGCGACGGCAAGGGCGAGATCG ACGACATCGACAACCTCGGCAACCGCCGCGTCCGTTCGGTCGGCGAGCTGATGGAAAAC CAGTACCGTCTCGGCCTGCTGCGCATGGAGCGCGCGATCAAGGAACGTATGTCCTCGATC GAGATCGACACGG
HFB1	HF - Soya Soil	100% identity to: • Rhizobium leguminosarum strain Tp73_4 • Rhizobium leguminosarum bv. viciae strain 3841	>HFB1 TCCGCATCCTGCGCAAGGACGACATTCTGGCCGTGGTCAGGATGCTGGTCGAACTGCGC GACGGCAAGGGCGAGATCGACGATATCGACAATCTCGGCAACCGCCGCGTCCGTTCGGT CGGCGAGCTGATGGAAAACCAGTACCGTCTCGGCCTGCTGCGCATGGAGCGCGCGATCA AGGAACGCATGTCCTCGATCGAGATCGACACGGT
HFB2	HF - Soya Soil	100% identity to: • Rhizobium leguminosarum strain Gr 12/7	>HFB2 CCGCATCCTGCGCAAGGACGACATTCTGGCCGTGGTCAGGATGCTGGTCGAATTGCGCG ATGGCAAGGGCGAGATCGACGACATCGACAACCTCGGCAACCGCCGCGTCCGTTCGGTC GGCGAGCTGATGGAAAACCAGTACCGTCTCGGCCTGCTGCGCATGGAGCGCGCGATCAA GGAACGCATGTCCTCGATCGAGATCGACACGGT

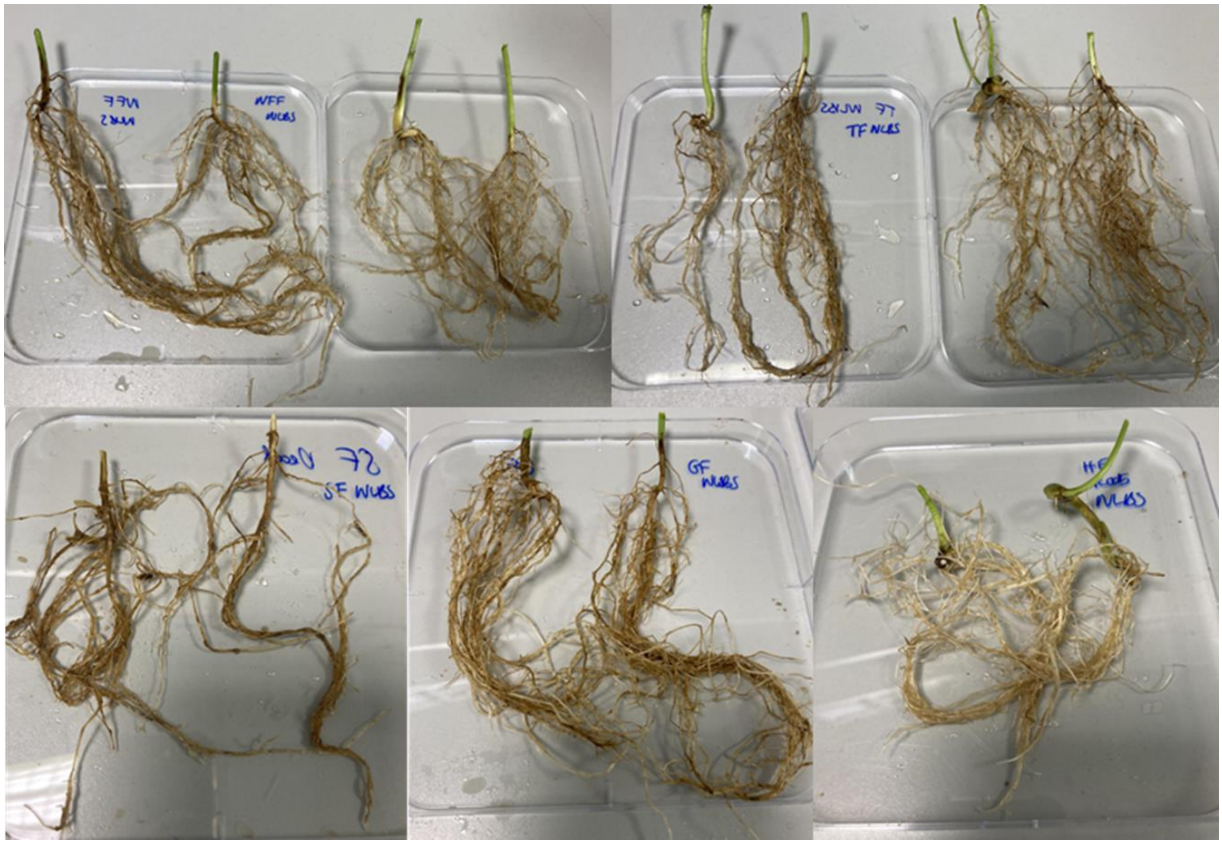

Figure S1. Photos of soybean plant roots grown in cereals soil from sites NFF (top left), TF (top right), SF (bottom left), GF (bottom middle), HF (bottom right). Roots were gently washed to remove soil and no root nodules were found.

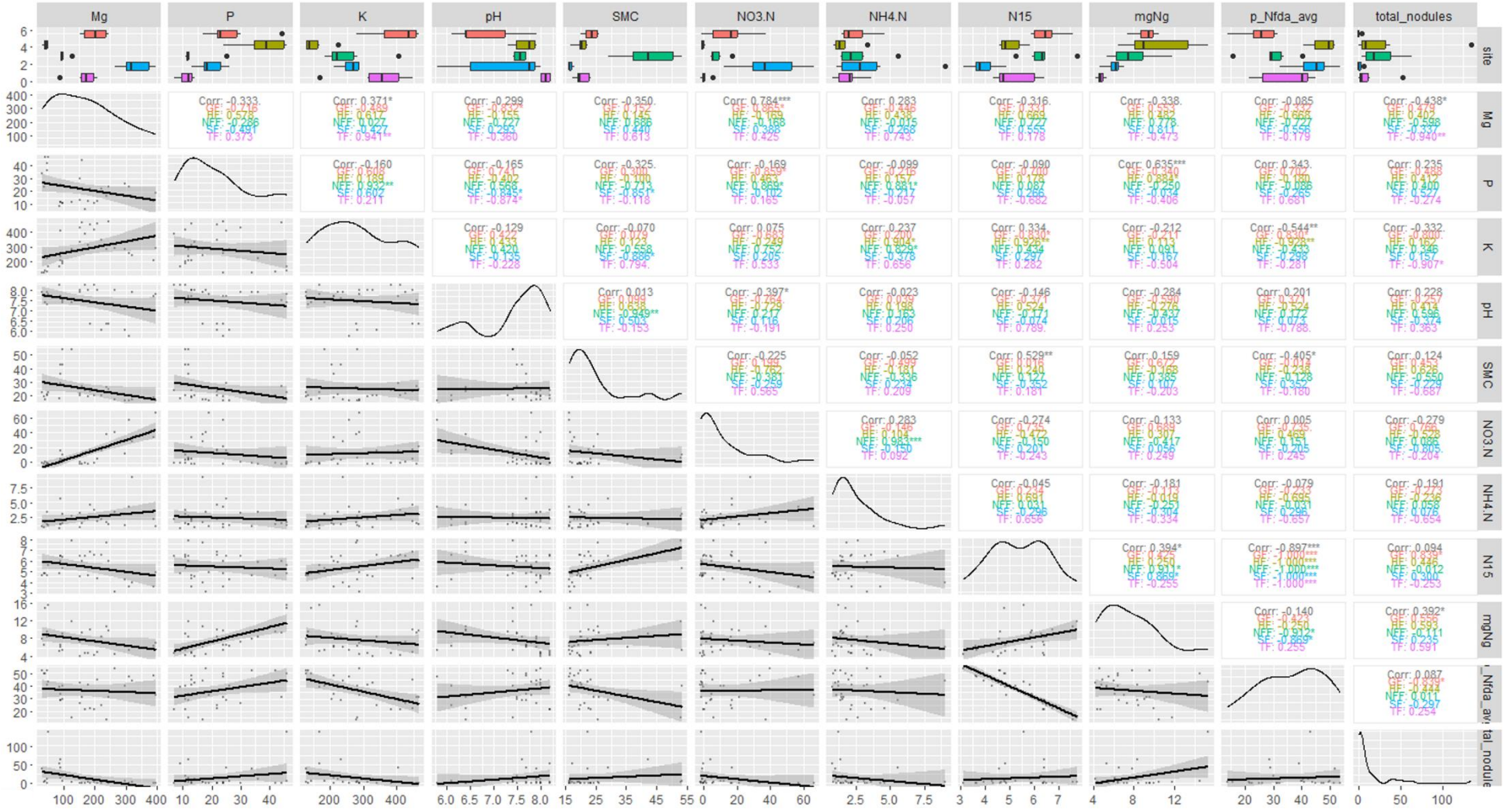

Figure S2. Pearson correlation matrix for soil traits and plant nitrogen contents. Correlation for whole dataset in black, including correlation line with confidence intervals (grey) *n* = 30. Correlations by field site in colours (*n* = 6), red = GF, gold = HF, green = NFF, blue = SF and purple = TF. Stars denote statistical significance of correlation, * = p < 0.05, ** = p < 0.01, *** = p < 0.001.

5.8 References

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Chapter 6: Discussion

6.1 Summary

Inoculation of non-native legume crops with exotic rhizobia is necessary to introduce biological nitrogen fixation (BNF) to a new area, however the impacts of inoculation on recipient soil communities are not well understood. Interactions between inoculants and resident soil communities can influence symbiosis outcomes, with knock-on impacts on crop yields. Optimising soybean (*Glycine max* L. Merr) inoculation strategies could aid soybean production in the UK, which would reduce reliance on imports. Effective rhizobia inoculation can also reduce chemical N inputs for legume growth. Thus, improved soya growth and inoculation in the UK could lead to a more sustainable soybean production for UK consumption. In this thesis, the effects of inoculating non-native rhizobial symbionts on soil communities and temperate soybean cultivars were investigated. Results showed that exotic rhizobial inoculation altered bacterial communities transiently in the short term (within one growth season), but there was evidence that functional soya-BNF had persisted in the microbiome from previous inoculations. Further investigation of multi-species inocula effects on soybean biomass, highlighted that *Bradyrhizobium* symbionts dominated nodule occupancy over *Sinorhizobium fredii* in a simple environment and provided better host benefits for the temperate cultivar tested. When *Bradyrhizobium* symbionts were combined with a plant growth promoting rhizobacteria (PGPR) consortia, some improved plant traits were observed and a novel beneficial association between *Bradyrhizobium* and the PGPR strain *Rhizobium languerre* PEPV16 was discovered. Finally, the potential for inoculant evolution during the first introduction of soybean across three UK farm sites was assessed, revealing strain variation within nodules, where inoculants may have diversified both pre- and post- introduction. Taken together, these results further our understanding of both the ecological and evolutionary impacts of exotic rhizobial inoculation on soil communities, with knock- on impacts for soybean yields. Here, these results are discussed within the wider

context of tailoring inoculants for soya BNF in the UK, the legacy of inoculants and their evolution, with future research areas outlined.

6.2 The potential to diversify soya inoculants

Currently, soybean inoculant products are dominated by *Bradyrhizobium* symbionts, however, research in this thesis also explored the potential of *Sinorhizobium fredii* symbionts for temperate varieties. This was explored due to *S. fredii'*s advantage in alkaline soil conditions (Temprano-Vera *et al*., 2018; Tian *et al*., 2012), and the overlap in the suitable soya growing area and alkaline soils in the UK (refer to Chapter 1, Figure 1). It is worth noting that in Chapter 2, *S. fredii* yielded a growth benefit compared to the N fertilised control in the soil without a history of soya inoculation, where there was a higher (but still neutral) pH and a smaller soya- nodulating *Bradyrhizobium* population than in the soya history soil. Previous research has shown that *Bradyrhizobium* symbionts usually outperform *Sinorhizobium* symbionts in most acidic - neutral pH soils (Ravuri and Hume, 1992) and particularly if *S. fredii* is inoculated into soils already possessing soya-nodulating *Bradyrhizobium* (Albareda *et al*., 2009a, 2009b), as was observed in the soya soil in Chapter 2. As soya- nodulating *B. diazoefficiens* and *B. japonicum* have now been introduced across areas of the UK, and in particular have shown evidence of high competitiveness, the likelihood of *S. fredii* inoculation effectiveness in UK soils may be low. However, comparison of inoculants performance in alkaline soils (>8 pH) was not directly tested in this research. Future research testing the application of *S. fredii* in UK agricultural alkaline soils with and without a history of inoculation would provide further information if plant yield benefits can be made. In recent years the exploration of utilising native legume symbionts as potential soya symbionts has highlighted that some novel associations can arise (BioVox, 2021; Bromfield *et al*., 2017; Van Dingenen *et al*., 2022), but it is worth noting that this is in areas with a longer history of soya growth and inoculation. Therefore, introduced inoculant strains may have had a long period in the soil microbiomes, with increased interactions with native

communities. Research is needed into native *Bradyrhizobium* communities in the UK as they are currently completely undescribed and the potential for successful horizontal gene transfer (HGT) of the symbiosis genes between inoculant and native *Bradyrhizobium* may be more likely to occur due to genetic adaptations and accommodations for symbiosis integrative conjugative elements (ICEs) (Weisberg *et al*., 2022b, 2022a).

Diversifying rhizobia inocula with beneficial PGPRs could increase the breadth of beneficial functions provided to legume crops. However, there is varied evidence for the effectiveness of PGPR inoculation and survival of introduced PGPR inoculants in soil microbiomes. For example broad acting PGPR *Azospirillum* inoculants are known for providing increases in crop yields, but are not persistent in the environment, particularly if host plants are removed (Bashan *et al*., 1995). A PGPR strain that is beneficial for one crop type, may not be beneficial for another, and therefore may not be selected and maintained by the plant holobiont (Hartmann *et al*., 2009; Venkateswarlu *et al*., 1997). The potential crop benefits that could be acquired from microbial inoculation can be diminished by certain agricultural practices, such as incompatible crop rotations (Koyama *et al*., 2022), fertilisation (Weese *et al*., 2015), tilling and fallow soil periods (Liu *et al*., 2021), alongside the reduced capacity of modern crop cultivars to form symbioses (Porter and Sachs, 2020). Thus, inoculation is necessary to introduce specific traits to certain environments (e.g. soya BNF to the UK), but a more holistic approach to crop production post inoculant introduction could help maximise the benefits of inoculation.

6.3 Competitiveness and persistence of inoculant strains

A notable finding in Chapter 2, was the persistence and spread of rhizobia inoculants. Previous soya growth and inoculation on the Kent farm had established a soil population of soya-nodulating *Bradyrhizobium*, which was able to provide plant benefits, but plant yields were not increased by not re-inoculation. This poses interesting questions about the impacts

of re-inoculation and whether it is necessary after the functional trait has been introduced to soil microbiomes. Soya-nodulating *Bradyrhizobium* strains have previously been found to persist in the soil microbiome 20 years after they were introduced to soils in Poland (Narozna *et al*., 2015) and 30 years post introduction in Brazil (Giongo *et al*., 2020). This is supported by evidence in other European soils (Brunel *et al*., 1988; Griebsch *et al*., 2020; Obaton *et al*., 2002) although longevity of inoculants has been found to be influenced by soil type (Revellin *et al*., 1996) and in some cases the time since soya was last grown (Halwani *et al*., 2021). Furthermore, the spread of *B. japonicum* from previously inoculated soils to un-inoculated, has also been documented (Mason *et al*., 2016; Mendes *et al*., 2004; Vargas *et al*., 1994). There are several contributing factors that may explain this spread and persistence, *Bradyrhizobium* are a diverse (Avontuur *et al*., 2019) and abundant clade of soil bacteria globally (Delgado-Baquerizo *et al*., 2018), with high saprophytic capability that occupy varying niches, for example the widespread dominance of nonsymbiotic *Bradyrhizobium* has been found across North American soils (VanInsberghe *et al*., 2015). The *Bradyrhizobium* inoculant strains SEMIA 5080 and SEMIA 5079 have a high proportion of genes related to amino acid and carbohydrate metabolism, and in particular *B. japonicum* SEMIA 5079 has more genes involved in secondary metabolites, nutrient transporters, iron-acquisition and auxin metabolism, which may further explain its success in soils (Siqueira *et al*., 2014). Traits that make a good inoculant, such as competitiveness in the rhizosphere, may also increase the likelihood of dispersal ability and survival, beyond the intended range or timescale (Jack *et al*., 2021). Mendes *et al*., (2004) noted that the inoculation of *B. japonicum* SEMIA 5079 established an ‘extremely unfavourable situation’, for the introduction of new inoculant strains, as it would outcompete them for nodule occupancy despite repeated inoculation with other strains (including SEMIA 5080). This means that if higher quality rhizobia strains are discovered and inoculated in the UK, they may get outcompeted by SEMIA 5079 for nodule occupancy. Positive soybean yield responses to re-inoculation have been observed in Brazil (Hungria *et al*., 2006, 1996; Vargas *et al*., 1994), but studies in North America have shown a lack of soybean yield response to re-inoculation

(De Bruin *et al*., 2010; Klubek *et al*., 1988). De Bruin *et al.*, (2010) evaluated re-inoculation of soybean in 73 field trials across midwest North America and found only 6 trials had a positive yield response to re-inoculation. Further research is needed in temperate areas but may suggest an alternative method to re-inoculation with the same 'elite' inoculant strains is needed for enhancing BNF potential.

6.4 Inoculant evolution - can we use it to our advantage?

The evolution of inoculant strains during the first growth season of soybean was found in Chapter 5. Polymorphic strain populations were observed within field grown nodules after their introduction to the soil four months prior. Surprisingly, around half of the mutations (~50 - 60%) were present at all field sites, suggesting a diverse intraspecific inoculant was introduced. As the nodulation step in the symbiosis is a population bottleneck of the potential pool of symbionts in the soil (Doin de Moura *et al*., 2023), we may expect a larger group of symbiont variants in the rhizosphere. This highlights interesting aspects about inoculant production and the eco-evolutionary processes it initiates. The idea of 'one size fits all', 'elite' inoculant strains that; remain genetically stable both *in vitro* and *in situ*, are beneficial in all soil environments and only persist until the desired function is carried out, is unsurprisingly unattainable (Kaminsky *et al*., 2019). Some strains that work well in one soil environment, may not in another, there is evidence of this in Chapter 5, where two out of five field sites exhibited poor nodulation. The diversity generated by inoculant production could increase the chance of introducing a beneficial strain in a given environment (Fields *et al*., 2021), which may be a good starting point and particularly useful when background variation is very low. However, mutations that likely occurred during inoculant production process are perhaps unlikely to confer benefit in the complex heterogeneous soil environment or *in planta*. Research in Chapter 5 was only a snapshot of the communities at an early time point; follow up analyses of these communities after successive seasons could highlight whether any mutations become fixed in the populations or form part of beneficial/

maladaptive mutational cohorts (Doin de Moura *et al*., 2023). Evidence suggests that a prolonged period in the soil may result in adaptation to the local edaphic conditions (Alves *et al*., 2003; Batista *et al*., 2015; Burghardt *et al*., 2019; Hungria *et al*., 2006; Santos *et al*., 1999), and if followed by subsequent rounds of host plant selection, co-evolution can result in more beneficial symbionts (Batstone *et al*., 2020; Doin de Moura *et al*., 2023; Quides *et al*., 2021). Locally adapted symbionts can also arise via HGT of the symbiosis genes from inoculant strains into native strains (Colombi *et al*., 2023; Hill *et al*., 2021). HGT allows evolution to occur on ecological timescales (Wardell *et al*., 2022), which in turn can feedback onto plant yields and thus subsequently drives the ecology and evolution of symbiont populations, exhibiting strong plant-soil feedbacks (terHorst and Zee, 2016). Although the mobility, modularity and flexibility of the MGEs that carry rhizobial symbiosis genes can result in cheater strains arising at the individual level, it is also hypothesised to maintain the function of symbiotic nitrogen fixation at a population level (Weisberg *et al*., 2022a), *i.e.* one legume's [genotype x rhizobia genotype x symbiosis MGE] treasure, is another legume's trash. Therefore, mutualism diversity is maintained within the environment (Heath *et al*., 2022; Weisberg *et al*., 2022a), where the generation of diverse symbionts may even be promoted in the rhizosphere (Ling *et al*., 2016; Remigi *et al*., 2014). The inherent nested nature of rhizobial symbioses suggests that a co-evolutionary approach could optimise introduced rhizobial symbionts to local conditions (Batstone, 2021).

6.5 Future directions

Taken together, the results from this thesis indicate that exotic rhizobial inoculation can impact soil microbial ecology and evolution. This can have impacts on the growth and yield of soybean grown in the UK. Research within the field highlights the benefits of co-evolution on rhizobial symbiont quality. Therefore, one way to optimise soya-BNF in UK soils could be to conduct field trials, inoculating temperate adapted cultivars with exotic inoculant strains once, to introduce the trait, and then growing soybean multiple times within these field trial

areas to allow plant hosts to exert selection on symbiont populations, aiding adaptation to the temperate genotypes, in novel soil environments. Trials could be conducted in various soil types and across climatic conditions, *e.g.* trials in Scotland may select for cold adapted symbionts, resulting in 'locally elite' strains which could be used going forward as more tailored inoculants. Desired trait outcomes should be defined, for example, enhanced seed yield and protein content may be the aim, to achieve soya suitable for human consumption, therefore it is important to grow crops to the desired growth stage, as many studies still only focus on early growth traits. Alternatively, improved host discrimination and more efficient sanctioning may be the desired outcome, therefore legume breeding programmes should include inoculation with a mix of rhizobial strains that vary in N_2 efficiency, as host discrimination works best when a high N_2 fixing strain is present (Denison, 2021; Westhoek *et al*., 2021). Other agricultural practices will need to be considered, for example not applying N fertiliser to trial plots will ensure plants are reliant on symbionts, and that reduced symbiotic traits do not inadvertently evolve. Other agronomic practices of growing interest, such as those in regenerative farming practices, could be incorporated to ensure realistic conditions for symbiont evolution. Follow up studies using extracted symbionts from the nodules of these field trials could be used to confirm their symbiotic efficiency *ex situ* and their potential as inoculant products. Inoculation research often starts by assessing a panel of bacteria on plant traits in ecologically irrelevant, sterile pot environments, and then scaling up to the field trials, where usually inoculant strains fail to survive and provide the desired function (Denton *et al*., 2002). Since inoculation of exotic soya- rhizobia has been an undertaken practice for years in the UK, we can utilise the power of environmental and host selection, flipping the process on its head. Starting with field trials to select for ecologically competitive symbionts, where repeated host exposure can increase the pool of efficient N_2 fixers. There is strong evidence that recurrent monitoring, strain extraction and testing of isolates can result in beneficial inoculant strains, a prime example is the inoculant industry in Brazil (Alves *et al*., 2003). This has produced inoculant strains that are competitive and appear to work across a range of soils, however if soya is going to become a commercial

reality in the UK to meet the growing demand, fine-tuning symbionts to local conditions is a necessity to optimise the potential of soya- BNF in the UK.

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Appendix

Soybean fields in Lincolnshire & Cambridgeshire, UK.

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Review

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Why are rhizobial symbiosis genes mobile?

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Rhizobia are one of the most important and best studied groups of bacterial symbionts. They are defined by their ability to establish nitrogen-fixing intracellular infections within plant hosts. One surprising feature of this symbiosis is that the bacterial genes required for this complex trait are not fixed within the chromosome, but are encoded on mobile genetic elements (MGEs), namely plasmids or integrative and conjugative elements. Evidence suggests that many of these elements are actively mobilizing within rhizobial populations, suggesting that regular symbiosis gene transfer is part of the ecology of rhizobial symbionts. At first glance, this is counterintuitive. The symbiosis trait is highly complex, multipartite and tightly coevolved with the legume hosts, while transfer of genes can be costly and disrupt co-adaptation between the chromosome and the symbiosis genes. However, horizontal gene transfer is a process driven not only by the interests of the host bacterium, but also, and perhaps predominantly, by the interests of the MGEs that facilitate it. Thus understanding the role of horizontal gene transfer in the rhizobium–legume symbiosis requires a 'mobile genetic element's-eye view' on the ecology and evolution of this important symbiosis.

This article is part of the theme issue 'The secret lives of microbial mobile genetic elements'.

1. Introduction

Rhizobia are defined by their ability to form intracellular, nitrogen-fixing infections in a broad range of plant hosts. This trait is highly complex and often tightly coevolved with the specific plant hosts they inhabit. One of the most surprising features of the rhizobial symbiosis is that, despite its complexity, the genes that underlie this defining characteristic are not embedded within the bacterial chromosome. Rather, they are encoded on mobile genetic elements (MGEs). Evidence both from experimental work and from phylogenetic comparisons, shows that many of these 'sym elements' are indeed able to transmit horizontally between bacterial hosts and that this is happening in some populations on a rapid—i.e. ecological—time frame. Other elements meanwhile show a strong fidelity to their host genomes and have lost the capacity to move independently.

The mobility of symbiosis genes is, at first glance, unexpected. Unlike the majority of bacterial accessory traits, nodulation and nitrogen fixation are hugely complex traits involving collaboration of a large suite of genes (*nod*, *nif*, *fix* and in some instances *fdx*) that orchestrate a complex series of events. Rhizobia must respond to and communicate with their specific plant hosts, infect and form intracellular colonies within plant nodules (controlled by *nod* genes) and then undergo sophisticated cell differentiation in order to devote cellular metabolism to the highly energy-intensive process of nitrogen fixation. Transfer of the symbiosis cassette risks breaking up these collaborative genes,

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as well as leaving behind any beneficial adaptation on the chromosome and other replicons. In addition, the transfer of symbiosis genes between bacteria is likely to be costly to the bacterial donor. Conjugation—probably the main route of symbiosis gene transfer—is an energy and time consuming process in itself but will also result in the creation of more competitors for the donor bacteria. If establishing a symbiosis within the plant is the bacterial equivalent of winning the lottery, then transfer of the symbiosis genes required for a given host is akin to handing out lottery tickets.

However, the dynamics of symbiosis genes in rhizobial populations is not under the control of the bacterial cells that host them. Rather it is driven by the MGEs that encode and carry them. Genetic elements with the ability to transmit to new hosts have—to varying degrees—their own evolutionary interests on which selection can act, sometimes to the detriment of the bacterial host they inhabit [1]. Thus the rhizobium–legume symbiosis should in fact be seen as a tripartite interaction between the plant, the bacteria and the MGEs that carry the functional trait [2]. In this review, we will examine the world of the sym element, asking two central questions: how mobile *is* the symbiosis, and what forces shape mobility among sym elements?

2. How mobile is symbiosis?

Mobility of the symbiosis trait can be observed through patterns of symbiosis gene distribution within and between rhizobial clades, as well as through examination of the specific MGEs that carry them. Overall the evidence points to widespread mobility among all of the major clades of rhizobia, but the level of mobility varies widely, suggesting alternative evolutionary strategies across species and between sym elements themselves.

(a) Evidence of sym gene transmission across rhizobia

Incongruence between the evolutionary history of sym genes and that of bacterial housekeeping genes (figure 1) has provided extensive evidence for the effect of sym gene mobility on rhizobial evolution and population structure. This literature has been extensively reviewed by [3], revealing a pattern of rare but significant transfer across large genetic distances, but far more frequent exchange among more closely related strains, within genera and species. For example, one early study of rhizobia from three genera (*Sinorhizobium*, *Rhizobium* and *Mesorhizobium)* showed widespread transmission within genera, but very little evidence of transfer between these larger clades [4].

Many examples of recent sym gene transfer stem from the introduction of legumes into novel environments through agriculture, which requires the simultaneous introduction of their compatible rhizobial symbionts. Subsequent mobilization of the crop-specific symbiosis genes from introduced strains into native strains and species appears common. An early example of this process was observed in New Zealand, where the inoculant *Mesorhizobium japonicum* strain R7A was co-introduced with the forage crop *Lotus corniculatus*. Seven years later, diverse *Mesorhizobium* strains isolated from *L. corniculatus* nodules harboured symbiosis genes identical to those of the original inoculant, strongly suggesting transfer of the symbiosis region into native *Mesorhizobium* strains [5,6]. A similar phenomenon has since been observed

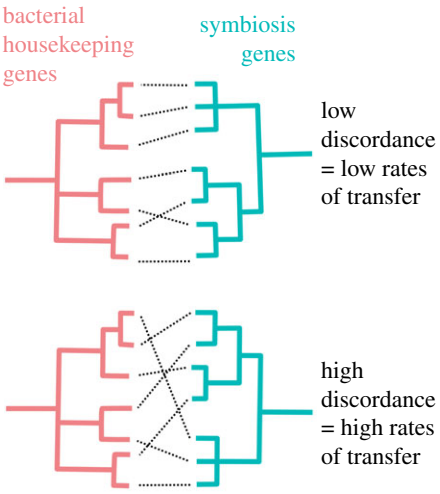

Figure 1. sym Gene transfer can be inferred from the level of discordance between phylogenies of bacterial housekeeping genes and sym genes. (Online version in colour.)

repeatedly across many hosts and geographical areas; in *Mesorhizobium* nodulating *Biserrula pelecinus* (a pasture legume) in Australia [7], in *Ensifer* nodulating soya in Brazil [8] and in *Rhizobium* symbionts of white clover (*Trifolium repens*) in China [9]. These examples demonstrate both the mobility of symbiosis genes and the importance of gene transfer in the evolution of the rhizobia–legume symbiosis. Mobilization allows the pairing of plant-specific genes with locally adapted bacterial genotypes, creating locally adapted symbionts, which facilitates range expansion of the legume host [10].

However, evidence for mobilization is not universal. *Mimosa* symbionts in Mexico, predominantly *Rhizobium*, and in Brazil, predominantly *Burkholderia*, both show co-divergence of bacterial chromosome and sym genes suggesting a stable evolutionary history between plants, symbionts and their sym genes [11,12]. In Uruguay, however, where *Mimosa* species are nodulated by *Cupriavidus*, incongruence suggests transfer *is* important [13]. Among published studies, therefore, evidence for regular mobilization is rife and examples can be found for every major clade of rhizobia studied [3].

However, while mobilization clearly occurs, it is difficult to estimate the rate of transfer within populations. Insights can be gained from studies of individual populations. In one study, a population of *R. leguminosarum* isolated from nodules of two hosts—clover and vetch—within 1 m^2 of soil revealed extensive incongruence between sym genes and the bacterial chromosome [14]. Different sets of sym genes are required for symbiosis with each of these two hosts, yet these were dispersed across the bacterial phylogeny, both across wide phylogenetic distances and between closely related strains, demonstrating that symbiosis gene mobility leads to regular reshuffling of host specificity within a population. Further studies of population-level variation are required to gain a clearer picture of the importance of ecological-scale sym element mobilization within rhizobial symbionts.

(b) Insights from the MGE ecosystem

Decades of research has built a picture of the MGE ecosystem within rhizobial genomes, revealing a wide diversity of

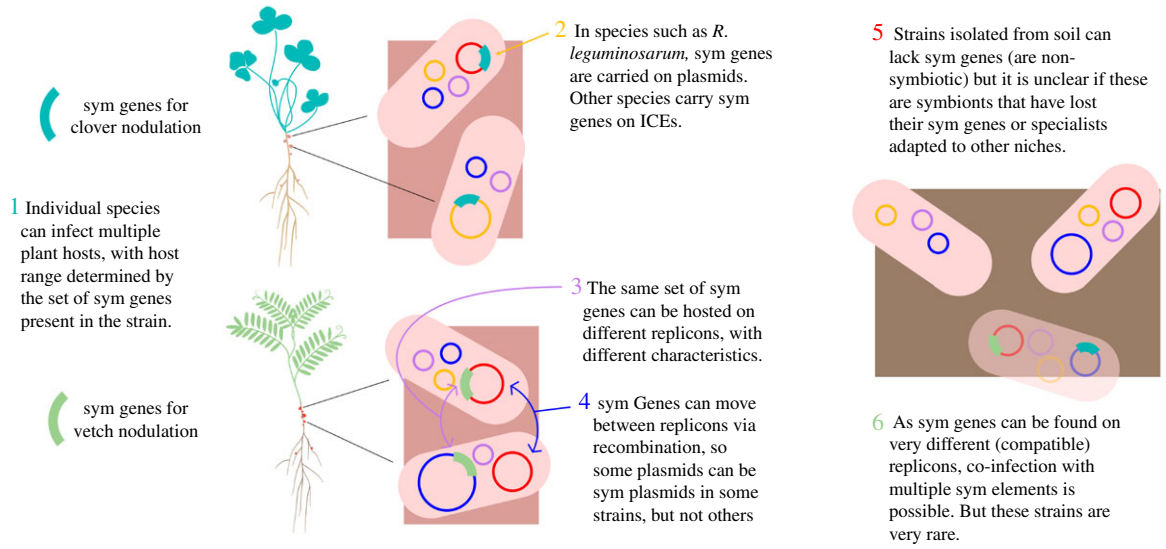

Figure 2. An illustration of sym plasmid diversity in species such as *Rhizobium leguminosarum*. (Online version in colour.)

MGEs that contribute to sym gene mobilization. For the most part, the major clades of rhizobia carry the core symbiosis genes (*nod*, *nif*, *fix* and, where present, *fxd*) on one type of replicon only. *Ensifer* (formerly *Sinorhizobium*), *Rhizobium*, *Cupriavidus* and *Paraburkholderia* typically carry sym genes on plasmids—pSyms [15–17]. *Mesorhizobium*, *Azorhizobium* and *Bradyrhizobium* predominantly carry sym genes on integrative and conjugative elements—ICEsyms—[6,18], which integrate into the genome at specific integration sites, similar to temperate phages, but can excise and initiate their own transfer through conjugation-like plasmids [19]. However, rare exceptions to this can be found. For example, *Bradyrhizobium* strains have been isolated carrying a symbiosis plasmid, rather than the more typical ICEsym [20]. The *nif* genes on this plasmid appear to have been derived from free-living N-fixing *Bradyrhizobium* strains, suggesting an independent origin for symbiotic N-fixation in this plasmid-carrying strain [21]. Such examples may well become more frequent with further sequencing.

Within each species and even within populations, multiple versions of symbiosis genes are typically present, creating a diverse sym element ecosystem (figure 2). This includes elements carrying different sym genes encoding instructions for nodulating different hosts (a group of bacteria that share a host range because they possess similar sym genes is called a 'symbiovar'). For example, *R. leguminosarum* populations can carry sym genes that enable symbiosis with clovers (symbiovar *trifolii*), Fabeae legumes (vetches, peas and faba beans; symbiovar *viciae)* or common bean (symbiovar *phaseoli*). Network analysis of plasmid genomes in *Rhizobium* suggests that plasmid clades primarily cluster by plant specificity, rather than bacterial host phylogeny [22].

Within a symbiovar the role of 'sym element' can be taken up by multiple distinct plasmids or ICEs [22,23] with very different characteristics in terms of mobility and genomic content. Whole genome sequencing of 196 strains of *R. leguminosarum* isolated from one host, white clover, across Europe revealed four different pSyms [23]. The pattern of pSym distribution suggests that these competing plasmids show very different rates of plasmid—and thus sym gene—transfer. While some pSyms showed fidelity to their host clades, others showed a strong signature of introgression—implying high rates of transmission [23]. Recombination can also lead to the mobilization of symbiosis genes between plasmids [24], potentially creating novel pSyms. Within *Rhizobium*, for example, sym plasmids for the most part appear to be distinct from other, non-symbiosis plasmids [22]—implying some co-adaptation with sym genes. However, several instances of sym genes on plasmids not universally associated with symbiosis have also been identified [22,23,25], implying transfer outside of the 'sym plasmid' pool. Indeed, the symbiosis genes have been suggested to have signatures of being readily mobilizable [26], suggesting that this genomic flexibility may well be adaptive.

Unsurprisingly, different sym elements will also lead to very different genes being in linkage with the symbiosis cassette. A wide variety of functional traits have been identified on pSyms beyond those encoded by the core sym genes, including those that are beneficial within the symbiosis—such as genes for citrate biosynthesis [27] or melanin synthesis [28], which is beneficial for managing redox conditions within the nodule [29]—as well as other environments—such as chemoreceptor genes [30], bacteriocins [22,23,25] and catabolic genes [31], which have been shown (in another plasmid) to be beneficial within the rhizosphere [32]. The pSym of *Ensifer meliloti* strain 1021, pSymA, is exceedingly large, carrying more than 1 Mb in excess of the symbiosis cassette itself. Systematic reduction of pSymA has revealed that just 63 kb (58 genes) of the 1.35 Mb plasmid is actually required for symbiosis [33]. However, strains carrying the 'minimum' plasmid containing these genes alone showed a significant reduction in their ability to compete with the wild-type strain for nodules [33]. Analysis of gene content suggests that the plasmid encodes numerous beneficial genes, e.g. those dealing with low oxygen environments encountered within the nodule [34], and metabolic genes that expand the range of carbon sources *E. meliloti* can metabolize [34,35].

Across replicons the capacity for mobilization is highly variable. Many sym elements carry the genes required to initiate their own transfer via conjugation, while others depend on mobilization by other MGEs. To date, four major classes of conjugative machinery have been described in rhizobial plasmids [36–38]. A list of examples of each type is provided in table 1. These conjugation machineries

Table 1. Examples of MGEs from each type of conjugation system. P, plasmid; I, ICEsym.

MGE	replicon type	details	references
type 1: quorum sensing (QS) mediated conjugation			
pNGR234a in *Rhizobium* sp. strain NGR234	P	Tra AHL mobilized plasmid. Conjugation rate estimated at 10^{-9}.	[39]
pRL1JI in *Rhizobium leguminosarum* sv. *viciae* 2483841	P	Well-studied pSym that is transferred at very high frequencies. QS is dependent on plasmid-free recipients.	[31]
		This plasmid seems to be made up of 3 modules: (1) a basic replicon with *repABC* genes and bacteriocin production and other genes that is similar to two other (unsequenced) plasmids pRL3JI and pRL4JI as well as transfer genes (Type I, QS regulated system); (2) a symbiosis region virtually identical to that in pRL10JI (from strain 3841); and (3) an extended region that looks like a catabolic region from pRL8JI.	[40,41]
ICE*Ml*SymR7A in *Mesorhizobium loti* strain R7A	I	ICE excision is highly controlled by TraR. Experimental derepression has shown that conjugation is functional but it has yet to be observed in wild-type strains. In addition, it has a second regulatory system, which also acts to further limit excision and transfer.	[42,43]
pSfr64b in *Ensifer/Sinorhizobium fredii* GR64	P	pSfr64b carries its own conjugative machinery but transfer is mutually dependent on a second plasmid, pSfr64a, for conjugation. Both plasmids carry regulatory genes that initiate conjugation of the other in response to QS molecules.	[44]
type II: RctA repression system			
pRetCFN42d in *Rhizobium etli* CFN42	P	pRetCFN42d carries its own conjugation machinery but this is heavily repressed and the environmental trigger is unknown. Transfer has been observed within nodules.	[45]
		This plasmid can also exploit other transfer machineries—mobilization has been shown to occur via integration and mobilization of the class I QS-induced plasmid p42a.	[46,47]
pSymA in *Ensifer/Sinorhizobium melliloti* strain 1021	P	Large (1354 kb) conjugative plasmid. Transfer has yet to be observed in the laboratory although there is evidence for transfer within nodules.	[33,34,48]
		63 kb region that contains the key symbiosis genes (*nod, nif* and regulatory genes).	
type III: mobilizable plasmids			
pRleVF39d in *Rhizobium leguminosarum* VF39SM	P	sym Plasmid carrying a chemotaxis gene.	[30]
pRL10JI in *Rhizobium leguminosarum* 3841	P	Plasmid carries a compact approximately 60 kb symbiosis gene cassette that is flanked by inverted repeat regions, suggesting the sym genes may be readily mobilizable.	[26]
type IV			
(type IVa) pRleVF39b in *Rhizobium leguminosarum* VF39SM	P	Plasmid carries the distinct type IVa conjugation system containing a small relaxase gene (*traA*) producing a shorter TraA protein, amongst other differences to the above systems. Mutagenesis studies highlighted the importance of *trcA-F* in conjugative transfer and alleviation of the repressor TrbR.	[37,38]
(type IVb) pSmed03 in *Ensifer medicae* WSM419	P	Plasmid carries the distinct type IV relaxase group (MOBP0) but clusters on a separate branch from type IVa systems.	[38,49]

(*Continued*.)

Table 1. (*Continued.*)

MGE	replicon type	details	references
alternative conjugation mechanisms			
ICEAc in *Azorhizobium caulinodans*	I	An 87.6 kb sym ICE found to excise and transfer in response to the host plant flavonoid naringenin. Increased transfers were also found after exposure to non-host plants, highlighting the rhizosphere as a promotive environment for HGT events.	[18]

effectively underlie crucial life-history traits for the MGE and consequently the conditions under which they are expected to be transferred.

— Type I elements are generally (but not exclusively) regulated by quorum sensing (QS) molecules AHLs (*N*-acyl-homoserine lactones). Consequently, conjugation is induced at high population density, although the details of the regulatory networks that control these systems vary. For example, in pSym pRL1JI of *R. leguminosarum*, conjugation occurs at high rates and is fine tuned to respond specifically to the presence of pRL1JI-free cells—i.e. potential recipients—rather than high population densities generally. The plasmid carries a repressor of AHL biosynthesis, eliminating AHL expression from existing carriers [40,41]. Non-carriers meanwhile produce AHLs; thus conjugation is induced when high densities of non-pRL1JI carriers are present in the environment. The ICEsym of *Mesorhizobium loti* strain R7A, on the other hand, has a highly controlled regulatory system induced through AHL [42] but also controlled by a second regulatory system that further fine tunes activation, limiting ICE excision and transfer within the population [43,50].
— In type II elements, conjugation is under the control of RctA, a repressor of the *virB* operon, required for conjugation. Very little is known about the environmental stimulus that alleviates RctA repression, suggesting that conjugation is limited to environments that are challenging to reproduce in the laboratory. For example, in pSym pRetCFN42d (*Rhizobium etli)* RctA repression can be experimentally relieved and transfer induced, showing that transmission is active, but the exact trigger cannot be identified [46,51]. However, recent work has demonstrated that pRetCFN42d transfer occurs within root nodules [47], suggesting that conjugation is tuned in yet unknown ways to the root environment.
— Type III elements, such as *R. leguminosarum* sv. *viceae* pSym pRL10JI, are not able to self-mobilize as they lack genes required for mate pair formation, but have retained the genes required for DNA transfer and replication. Although they are unable to initiate conjugation themselves they can, in theory, hitchhike with other conjugative plasmids within the cell, although this has yet to be observed.
— More recently, a fourth class of conjugative plasmids (type IV) has been identified, which uses a distinct repression pathway. This type of system is present on pSyms, such as pRL5JI in strain TOM [37], and non-sym plasmids, in a wide array of different rhizobial species [38,52].
— Furthermore, distinctive conjugation machineries can be found in the rhizobial ICE replicons. Mobilization of the ICEsym of *Azorhizobium caulinodans* (ICE^{Ac}) is induced in the presence of plant flavonoids excreted from the roots of the host plant [18]. Conjugation is under the control of a homologue of *nodD*, which initiates nodulation. Thus ICE^{Ac} conjugation is explicitly linked to the conditions in which the sym genes would be beneficial.

These divergent conjugation types group both by mechanism and phylogenetically, representing divergent clades of conjugation genes [38]. Single rhizobial strains can play host to multiple types of these elements [38], and sym elements can be drawn from multiple types within taxa [23]. In some cases sym elements can themselves use multiple pathways—type II *R. etli* pSym pRetCFN42d, for example, has been shown to mobilize via co-integration with the cohabiting, type I QS plasmid pRetCFN42a [45]. Consequently, sym element transfer will depend on both the inherent conjugation rate of the sym element and the conditions required to initiate transfer—through cell density, environmental cues or, for type III elements, the community of MGEs that share the same host.

3. What forces may act to maintain mobility?

Horizontal transfer of symbiosis genes is clearly important to the evolutionary history of the rhizobium–legume symbiosis. Acquisition of sym genes was central to the origins of the major rhizobial clades [53], and has been shown to be key in legume range expansion [6–9,54]. However, the utility of such rare events is not sufficient to explain what forces maintain selection for mobility of this crucial trait. Symbiosis gene transfer has no clear benefit for the bacterial donor; conjugation events are energetically costly [55] and the formation of new symbionts in the community only increases competition for plant hosts. Rather, the dynamics of symbiosis mobility are best understood from the perspective of the MGEs that drive gene mobilization. Consequently, it can be expected that sym elements are under selection to maintain their mobility between bacterial hosts.

(a) Conditions that favour sym element mobility

(i) Heterogeneity in selection for symbiosis traits

Despite being the defining characteristic of rhizobia, the symbiosis trait is typical of bacterial accessory traits, in that

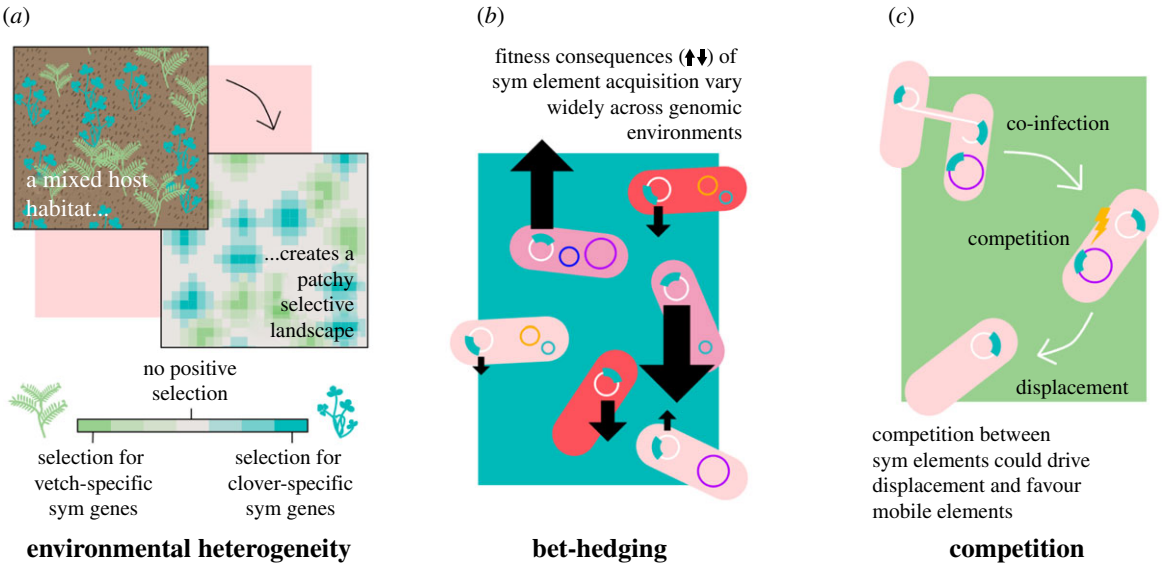

Figure 3. Conditions that favour plasmid mobility. (*a*) Plant hosts requiring different sets of symbiosis genes can exist in sympatry (e.g. clover and vetch nodulated by *R. leguminosarum* sv. *trifolii* and *viciae*, respectively). Plants may act as hotspots for selection on different sym genes with areas of no or low selection in between. (*b*) The same sym element can have different fitness/symbiotic qualities across different bacterial genotypes and in the presence of different co-infecting plasmids. Plasmid transfer therefore creates diversity of symbiotic function and plasmid fitness. (*c*) Co-infection of different sym elements may drive selection for mobility. Co-infecting sym elements could displace the existing sym element, explaining the lack of dual-sym plasmids. (Online version in colour.)

positive selection is both spatially and temporally heterogeneous. From a bacterial perspective, the distribution of host plants in natural ecosystems is extremely patchy. Plants infected by the same rhizobial species but different symbiovars (requiring different sym genes) often exist in sympatry. This creates a patchwork of positive selection for different plant-specific sym genes across a landscape (figure 3). For example, the clover and the vetch hosts of *R. leguminosarum* often co-occur in the same environment. Correspondingly, the associated *R. leguminosarum* population displays a high degree of exchange of pSyms encoding clover and vetch specificity [14]. In addition, plant demand for symbiotic partners will vary widely over time depending on their nitrogen requirements [56]. When nitrogen is available in the soil, or during periods of low growth when nitrogen is not required, nodules will senesce and their bacterial populations return to the soil [56]. Illustrating this, long-term supplementation of nitrogen through fertilizer can lead to reduced symbiont quality in resident rhizobia populations [57,58].

Intermittent positive selection has been shown to favour traits spread by horizontal gene transfer (HGT). In the absence of selection, genes can be lost through purifying selection. Mobility serves to counteract this loss through infectious transmission [59–61]. Intermittent positive selection can then stabilize MGE prevalence through selective sweeps carrying elements to high frequency [62] or via source–sink dynamics [63]. sym Plasmids in particular are known to be lost from laboratory strains through subculturing [64], suggesting that they may be readily lost from strains while free-living in the soil. Experimental curing of sym plasmids has, in some instances, been shown to be associated with increases in bacterial growth [65], suggesting that purifying selection may favour loss of plasmids from the population. Although it should be noted that pSym loss can also be associated with loss of other functions that may be beneficial in the rhizosphere [35,65], making the implications of plasmid loss context dependent. Natural rhizobia populations are repeatedly found to contain a significant proportion of strains that lack sym elements entirely. Outside the plant host, rates of Sym^- strains can be very high; one study in *Bradyrhizobium*, where symbiosis genes are encoded on ICEsyms, found approximately 50% of soil isolates lacked key symbiosis genes [66]. Another study in *Rhizobium* found that more than 97% of soil isolates were non-symbiotic [67]. Sym^- strains can even be isolated within plant nodules, demonstrating that positive selection for symbiosis is not necessarily consistent within host plants. In *Mesorhizobium*, approximately 16% of strains isolated from nodules lacked the symbiosis genes, creating symbiotic 'cheats' that benefit from the plant resources without providing nitrogen fixation services in exchange [68]. Indeed, it is clear that rhizobia strains have many 'other lives' beyond the role of the 'good symbiont' [69] in which sym genes may be superfluous or even detrimental. Analysis of *Bradyrhizobium* populations in and around *Lotus* plants found soil populations contained far higher diversity than plant-associated populations [70], suggesting a multitude of other niches in which rhizobia may specialize. For Sym^+ strains, demand from legume hosts represent spatial and temporal hotspots of positive selection for sym elements, which may act to favour sym element mobility.

(ii) Evolutionary bet-hedging

In diverse host populations, conjugation allows MGEs to sample alternative genomic environments (figure 3). This has been proposed as a mechanism for plasmid persistence in the absence of positive selection; transfer increases the likelihood of associating with a strain undergoing a selective sweep targeting other sites on the genome and thus carrying the MGE to high frequency [71,72]. In the presence of selection, however, conjugation can be thought of as akin to sexual recombination, reshuffling the genomic deck and potentially generating beneficial combinations [73]. Rhizobial

effectiveness can vary widely between strains within symbiovars, depending on the bacterial genomic background and also the resident MGE community and the plant genotype. Transfer of soya-specific pSyms between *E. fredii* strains, for example, created unpredictable patterns of host specificity across different soya bean cultivars [74]. Consequently, sampling novel bacterial host backgrounds through conjugation could benefit the sym element by increasing the probability of producing a more successful bacterial symbiont for locally specific plant host × environment combinations.

(iii) Intracellular competition

Finally, it is possible that competition between mobile sym elements may itself contribute to selection for mobility within the population. Nodulating populations can carry a wide diversity of sym elements, which can be drawn from very different incompatibility types, suggesting they are able to co-infect. In *R. leguminosarum*, for example, strains can be found with coexisting *potential* sym plasmids (i.e. non-sym plasmids that in other strains act as the pSym), suggesting compatibility between plasmid backbones [23]. Strains with multiple pSyms are rare, however, suggesting conflict between plasmids when they are performing the same function. Similar destabilization has been observed among co-infecting mercury resistance plasmids. In the absence of selection for a shared trait, co-infection of two plasmids carrying the same mercury resistance operon enhances plasmid stability [75]. However, counterintuitively, in the presence of mercury selection coexistence is destabilized and one plasmid is lost [75]. Most rhizobial genomes have been isolated from functioning nodules—i.e. from conditions in which symbiosis genes are under positive selection. It is possible, therefore, that co-infection of a bacterium with multiple sym elements is disruptive during infection, leading to the loss of redundant versions of the sym element (figure 3). Where this is the case, competition within the host may drive selection for sym element mobility—as more mobile genotypes will be expected to displace non-mobile genotypes over time through co-infection.

Intracellular competition between sym elements has been proposed as the driver of ICEsym evolution in *Mesorhizobium*, albeit with a very different outcome. Chickpea-nodulating *Mesorhizobium* strains carry a distinctive tripartite ICEsym that integrates and excises as one replicon, but when integrated undergoes a series of recombination events that divides the ICE replicon into three non-contiguous sections [76,77]. Haskett *et al.* [76] proposed that this organization gives the tripartite ICE greater resistance to competition from other ICEs, such that tripartite ICEs should be resistant to excision triggered by incoming competitors. Consistent with this prediction, an analysis of *Mesorhizobium* genomes revealed that monopartite ICEs were more prone to transfer compared with the tripartite ICEs, which show greater host fidelity [78]. In addition, it was noted that strains carrying multiple ICEsyms only carry monopartite and not tripartite ICEs [78]. Thus competition between sym elements appears to have contributed to the evolution of strategies to resist superinfection—in this case leading to competitive exclusion of one clade of elements over another.

(b) Strategies for minimizing the costs of mobilization

While sym element mobility may be beneficial, the process of HGT can be costly for both bacterial donor and recipient. As MGEs depend on their bacterial hosts for survival (via replication during cell division), the persistence of sym elements will also depend on reducing the costs imposed during transfer.

For the bacterial donor the act of conjugation is a costly endeavour. Conjugation is initiated by the conjugative element and requires the cell to invest in plasmid/ICE genome replication, conjugation pilus construction and the time required for transfer between host and recipient [55]. During this time the cell can become susceptible to phages which target the conjugative pilus [79]. Secondly, successful transfer requires that the recipient cell lacks a copy of the incoming element or an element sufficiently related to cause incompatibility, in the case of a plasmid. Some—though not all—ICEs require integration sites that are unoccupied, and plasmids cannot coexist if their replication or partitioning systems are too closely related [80].

Once transferred, MGEs can be highly costly to new hosts. This has been well documented for plasmid transfer in other systems and is often associated with significant growth costs due to a wide range of factors. These include the costs of plasmid maintenance and transfer, disruption to cellular regulation and antagonistic interactions with existing genes [81,82]. These costs can often be unpredictable, e.g. owing to interactions between incoming plasmids and MGEs already resident in the genome [83]. Over time, however, the cost of plasmid acquisition is likely to be resolved through compensatory mutations [84]. The success of transfer to a novel host will thus depend on the size of the initial cost, and the accessibility of compensatory mutations to relieve it [85]. Experimental transfer of sym plasmids into strains lacking sym elements has demonstrated that transfer can result in functional symbionts, with no detectable cost to symbiotic efficiency [67], but further work to understand the cost of pSym or ICEsym transfer to the bacterial cell is needed.

The tight regulation of sym element transfer is one mechanism by which these costs can be minimized. QS regulation means that transfer occurs under conditions of high population density, which are likely to occur within the rhizosphere. However, such QS systems could still be prone to 'misfiring'. The rhizosphere environment is likely to be enriched with sym plasmid carriers already, and may well not be the rhizosphere of the correct plant! Fine-tuning these mechanisms, for instance by specifically targeting non-carriers [40,41], or sensitivity to specific plant flavonoids [18], can reduce the probability of unsuccessful transfer events but these appear—for now—to be rare.

Successful establishment can also be increased through linkage with other beneficial traits beyond the core sym genes. Experimental curing of symbiosis plasmids is often associated with specific growth costs, such as loss of metabolic functions [35], bacteriocin production [22,23,25] and competitive ability [65], which could be disadvantageous in the rhizosphere. Linkage with functions not associated with symbiosis will increase the range of environments in which acquisition of a sym element can be beneficial and thus reduce the conditions under which plasmids may be lost.

(c) Modular genomes maintain mobility

Finally, the success of sym element transfer is also dependent on the integration and function of sym genes once acquired.

Box 1. Future directions for the evolutionary ecology of rhizobia MGEs.

What role does (co)evolution play in sym element transfer? Experimental transfer of sym elements suggests that the success of sym element mobilization varies widely with background. Transfer between closely related strains appears to incur little cost and often (though not always) results in a functioning symbiosis. By contrast, pSym curing frequently constrains bacterial viability. This could be explained by pre-adaptation to accommodating symbiosis genes as well as—in some cases—a wider variety of other non-symbiosis plasmids. Across large genetic distances, where the opportunities for co-adaptation are limited, sym element transfer is less successful and can require extensive adaptation to acquire only partial functionality. Evidence from other host–plasmid relationships suggests that some degree of adaptation—sometimes co-adaptation—of host or plasmid is the norm following MGE acquisition. Future studies are required to understand the role of pre-adaptation in sym element transfer and function and how this may constrain transmission through rhizobial populations.

Why are dual-sym rhizobia so rare? Many rhizobial populations are home to diverse sym elements which encode comparable functions, i.e. symbiosis with a specific host, but are not obviously incompatible. Yet strains carrying more than one sym element are rare. Are 'dual-sym' strains more common in soil environments—where their symbiosis functions are downregulated—and does nodulation lead to displacement of one element by the other?

How do sym elements mobilize through the rhizobial metapopulation? The legume symbiosis is just one of numerous niches that rhizobial populations inhabit, and studies suggest that sym-gene-carrying rhizobia may in fact be in the minority in the population as a whole. The vast majority of studies have focused on rhizobial strains isolated from plant nodules, but it remains unclear how sym elements are shared across the wider metapopulation. For example, are all rhizobia within a population potential sym element hosts, or are rhizobia occupying alternative niches maladapted to conversion to symbiosis via HGT?

Experimental approaches in sym element ecology and evolution. The rhizobium–legume symbiosis is one of the best studied mutualisms in the world, but there remains a great deal to understand about the rhizobial populations, as outlined above. Addressing these questions requires two key approaches: firstly a greater exploration of rhizobial populations beyond the nodule environment. Studies that have investigated these populations suggest that there is a great deal of diversity outside the host. Whole genome sequencing of these populations would reveal more about the structure of sym element populations in addition to that of the host. Secondly, use of evolutionary ecology techniques such as experimental evolution and competition experiments can help to explore the fitness consequences of plasmid transfer, and the downstream adaptations that are required to accommodate a new sym element into the genome. The use of such experiments in combination with molecular approaches can be a powerful tool to reveal the routes and barriers to sym transmission.

Bacterial accessory genes, i.e. genes prone to horizontal gene transfer, are highly diverse, encoding functions, such as resistance traits, virulence factors or novel metabolic functions, that are often made up of comparatively small operational units. The rhizobial symbiosis stands out as a particularly large and complex trait involving three or four sets of genes, typically arrayed together in an approximately 100 kb sequence, that control a series of processes culminating in nitrogen fixation. One interesting comparison for the symbiosis traits is among bacterial pathogenicity genes [86], which are likewise complex, large and well known to be transferred through horizontal gene transfer (HGT). Notably, like symbiosis genes, they provide the blueprint for infection of a eukaryotic host. In both cases, these complex gene cassettes are composed of smaller operational units that have become linked over time through selection [87,88].

There is also evidence to suggest that the symbiosis cassette operates as a (relatively) self-contained operational unit. Genes that are heavily integrated into gene networks are extremely costly to acquire as they are likely to lead to regulatory disruption [89]. Consequently, accessory genes typically have a relatively low level of transcriptional connectivity [90]. Analysis of regulatory cross-talk across the three replicons of *E. meliloti*, the chromosome, the symbiosis plasmid pSymA and the chromid pSymB (not actually a pSym, despite its name), showed a significant absence of cross-regulation, particularly between pSymA and other replicons [91]. Curing of the symbiosis plasmid resulted in very little transcriptional disruption across the rest of the genome [92]. In comparison, curing of the chromid led to differential expression in 8% of chromosomal genes [92]. A similar pattern has been observed in *R. etli*, where predicted connectivity between genes carried on all replicons was lowest for two plasmids, the pSym pRetCFN42d and pRetCFN42a, the plasmid known to co-transfer with the pSym [93]. Modularity of sym elements within the genome—and potentially symbiosis genes within their mobile replicons—demonstrates how such complex traits are able to maintain mobility in rhizobial populations. One counterpoint to this, however, is the existence of direct regulatory control between replicons in several known cases related to *fixNOQP* and *fixGHIS* genes [94–96]. In pRetCFN42d, expression of *fix* genes is regulated by genes on another, less mobile, plasmid, pRetCFN42f [95]. Dependence on these regulatory networks likely limits the range of hosts that can effectively use newly acquired symbiosis genes.

It is worth noting, however, that the *nod*, *nif*, *fix* and *fxd* genes of the symbiosis cassette—while essential for symbiosis—are far from the only genes used during symbiosis. Many other parts of the genome, both chromosomal and plasmid-encoded, collaborate to hone the symbiotic relationship between a bacterium and each host plant [97–99]. For this reason, transfer of the sym plasmid alone cannot create new rhizobial symbionts. Attempts to experimentally evolve novel nitrogen-fixing symbionts demonstrate that transfer of the symbiosis function to non-rhizobial hosts can be extremely challenging [100–102], implying that a significant level of pre-adaptation is required for successful utilization of the symbiosis genes. Guan *et al*. transferred the pSym of a *Mimosa* symbiont to the pathogen *Ralstonia*

solanacearum. The evolved 'symbiont' was able to initiate nodulation but not nitrogen fixation, despite repeated rounds of selection *in planta*. Indeed, close relatedness alone is not necessarily a guarantee of successful transfer. Transfer of symbiosis plasmids between the symbiont *R. leguminosarum* and more closely related *Agrobacterium* did not result in a functional symbiosis, even when multiple plasmids known to affect symbiosis were combined [103].

4. Conclusion and future directions

Horizontal transfer of symbiosis genes has played a foundational role in the origin of rhizobial symbionts and facilitates rapid adaptation of the symbiosis to new environments. Within populations, sym element exchange appears to be occurring on an ecological scale, generating diverse symbiont populations from which legume hosts can sample. Both the rhizobial symbionts that gain the functions and the plant hosts that depend on them can benefit greatly from this process, but control of conjugation rests predominantly with the MGEs that mediate sym gene transfer. Future work examining the evolutionary and ecological forces acting on these elements is therefore key to understanding the dynamics of this important symbiosis (box 1). Decades of detailed work has revealed a complex and diverse ecosystem of MGEs within rhizobial genomes as well as a meticulous understanding of—at least some of—the diverse mechanisms that underlie this process. The recent discoveries of novel conjugation machineries among rhizobial plasmids demonstrates that this diversity is far from understood—but provides a firm grounding for future work applying ecological and evolutionary perspectives to this intracellular community.

Data accessibility. This article has no additional data.

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