Microbiome profiling reveals that *Pseudomonas* antagonises parasitic nodule colonisation of cheater rhizobia in *Lotus*

Dissertation zur Erlangung des Doktorgrades der Naturwissenschaften

Doctor rerum naturalium (Dr. rer. nat.) an der Fakultät für Biologie der

Ludwig-Maximilians-Universität München

vorgelegt von
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München, August 2022

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Tag der Abgabe: 8 August 2022

Tag der mündlichen Prüfung: 18 November 2022

Eidesstattliche Erklärung

Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

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Erklärung

Hiermit erkläre ich, dass die Dissertation nicht ganz oder wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist. Ich habe nicht versucht, anderweitig eine Dissertation einzureichen oder mich einer Doktorprüfung zu unterziehen.

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Publications originating from this work

- Kabdullayeva T, **Crosbie DB**, Marín M. *Mesorhizobium norvegicum* sp. nov., a rhizobium isolated from a *Lotus corniculatus* root nodule in Norway. International Journal of Systematic and Evolutionary Microbiology. 2020 Jan 1;70(1):388-96.
- **Crosbie DB**, Mahmoudi M, Radl V, Brachmann A, Schloter M, Kemen E, Marín M. Microbiome profiling reveals that *Pseudomonas* antagonises parasitic nodule colonisation of cheater rhizobia in *Lotus*. New Phytologist. 2022 Apr;234(1):242-55.

Declaration/Declaration of contribution as co-author

Publication I: *Mesorhizobium norvegicum* sp. nov., a rhizobium isolated from a *Lotus corniculatus* root nodule in Norway

In this publication T Kabdullayeva conducted the ANI, phylogenetic analysis as well as the genome assembly and annotation. I performed the physiological and chemotaxonomy assays. T Kabdullayeva, M Marín and I all contributed to the writing process.

Publication II: Microbiome profiling reveals that *Pseudomonas* antagonises parasitic nodule colonisation of cheater rhizobia in *Lotus*

In this publication M Marín and I conceived the project. I performed plant inoculation, harvesting and phenotyping as well as isolating bacteria from nodules. I created the libraries for sequencing and processed the sequencing output. M Mahmoudi carried out SVM and network bioinformatics, A Brachmann and V Radl performed MiSeq sequencing. M Marín, M Mahmoudi and I contributed to the writing and manuscript preparation. All authors read and approved the final manuscript.

Signature of supervisor

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Dr. Macarena Marín

Abbreviations

°C	Degree(s) Celsius
ACC	1-aminocylcopropane-1-carboxylic acid
ANI	Average nucleotide identity
ANPR	*Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*
ANOVA	Analysis of variance
ASV	Amplicon sequence variant
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
bv.	Biovar
ca.	Circa
cm	Centimetre(s)
CLSM	Confocal laser scanning microscopy
cv.	Cultivar
EPS	Exopolysaccharides
FAB	Fabaceae medium
g	gram(s)
GA	Gibberellin
h	hour(s)
LB	Luria-Bertani broth
MLSA	Multilocus sequence analysis
min	Minute(s)
mm	Millimetre(s)
Nod factor	Nodulation factor
OD	Optical density
PCR	Polymerase chain reaction
PGPB	Plant-growth promoting bacteria
PERMANOVA	Permutational analysis of variance
PMM	Pseudomonas minimal medium
qPCR	Quantitative polymerase chain reaction
rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulfate
Sp.	Species
SVM	Support vector machine
TS	Tryptone soy
µL	Microlitre(s)
µm	Micrometre(s)
V3-V4	Variable regions 3-4
w/v	Weight/volume
wpi	Weeks post inoculation
YMB	Yeast-mannitol broth
YM	Yeast-mannitol

Summary

Nitrogen fixation is carried out inside nodules of legumes by symbiotic rhizobia. Rhizobia dominate the nodule microbiome, however, other non-rhizobial bacteria also colonise root nodules. It is not clear whether these less abundant nodule colonisers impact nodule function. In order to investigate the relationship between the nodule microbiome and nodule function as influenced by the soil microbiome, we used a metabarcoding approach to characterise the communities inside *Lotus burttii*, *Lotus japonicus* and *Lotus corniculatus* nodules from plants that were either starved or healthy, resulting from inoculations with different soil suspensions in a closed pot experiment. We found that the nodule microbiome of all tested *Lotus* species differed according to inoculum, but only that of *L. burttii* varied with plant health. Using a machine learning algorithm, we found that out of all the non-rhizobial bacteria inside the *L. burttii* nodules, amplicon sequence variants (ASVs) corresponding to *Pseudomonas* were the most indicative signatures of healthy plants. *Rhizobium* ASVs were the most indicative of a starved *L. burttii* plant nodule. Network analysis revealed that there were exclusively negative significant correlations between *Rhizobium* and *Pseudomonas* ASVs and both positive and negative correlations between *Pseudomonas* and *Mesorhizobium* ASVs. This was supported by a co-inoculation experiment on *L. burttii* that revealed fluorescently tagged *Pseudomonas* could co-colonise nodules formed by *Mesorhizobium*, but not those formed by *Rhizobium*. Further evidence for the potential plant benefit of *Pseudomonas* presence was seen in a co-inoculation with *Rhizobium* on *L. japonicus.* The number of ineffective nodules induced by the *Rhizobium* isolate was reduced when inoculated together with a *Pseudomonas* nodule isolate. The same reduction in ineffective nodules was not seen in *L. burttii*. These results support the hypothesis that legume nodule endophytes influence the overall outcome of the root-nodule symbiosis, albeit in a plant host-specific manner.

In addition, a novel *Mesorhizobium* species, *Mesorhizobium norwegicum,* was physiologically and chemotaxonomically characterised.

Introduction

1. The plant holobiont

Plants and microbes have coevolved for millions of years (Sprent, 2008). The sessile state of plants means that they must adapt to survive in changing environments. One proposed method of plant adaptation is the utilisation of surrounding microbes (Vandenkoornhuyse *et al.*, 2015). Bacterial evolutionary features such as horizontal gene transfer and the accumulation of mutations have facilitated their adaptation to many plant niches (Wiedenbeck & Cohan, 2011; Good *et al.*, 2017; Trivedi *et al.*, 2020; Compant *et al.*, 2021). The plant and all the microorganisms with which it associates, both mutually, parasitically as well as neutrally, are referred to as the plant holobiont (Lyu *et al.*, 2021). These microbes colonise the phyllosphere, the aboveground part of the plant, the endosphere, plant internal tissues, the rhizoplane, the root surface, and the rhizosphere, the soil region that is influenced by root secretions (Hassani *et al.*, 2018). Selection pressure from both the plant and other microbe-microbe interactions has likely led to the evolution of microbial communities that influence the fitness of the plant (Hassani *et al.*, 2018; Cordovez *et al.*, 2019). Bacteria that are part of the holobiont can contribute to plant fitness by aiding in disease suppression, nutrient acquisition, environmental stress tolerance and the promotion of beneficial associations with fungi (Garbaye, 1994; Mendes *et al.*, 2011; Rolli *et al.*, 2015; Van Der Heijden *et al.*, 2016). Many of these benefits are dependent on environmental pressures and others have been selectively evolved for their specific host (Berendsen *et al.*, 2012; Bulgarelli *et al.*, 2012). An example of a specific plant-microbe coevolution is root-nodule symbiosis, which occurs in nitrogen-limiting conditions when leguminous plants host nitrogen-fixing rhizobia endosymbiotically.

2. Root-nodule symbiosis

2.1 The mechanism of root-nodule symbiosis

The atmosphere is the largest reserve of nitrogen in the planet. Nitrogen is pivotal for plant growth (Leghari *et al.*, 2016) and legumes have evolved a system of attaining nitrogen with the help of bacterial partners. Gaseous nitrogen is unreactive; however, it can be made bioavailable to plants via catalytic reactions facilitated by diazotrophic bacteria (reviewed in Shin *et al.*, 2016). Root-nodule symbiosis is a process through which primarily leguminous plants, such as soybean and peanut, utilise diazotrophic soil bacteria, collectively called rhizobia. These bacteria provide nitrogen to the plant at the cost of being accommodated endosymbiotically (Venado *et al.*, 2020). The symbiosis begins when plant roots release flavonoids into the surrounding soil, which are perceived by rhizobia (Bolaños-Vásquez & Werner, 1997). In response the rhizobia produce lipochitooligosaccharide molecules, called

Nodulation (Nod) factors. These are perceived by the plant, which then triggers a signalling cascade that induces the plant to begin cell divisions in the root cortex and epidermal root hair curling (D'haeze & Holsters, 2002). These cortical cell divisions form into root organs called nodules which host compatible rhizobia that have infected the root through infection threads formed in the root hair or via intercellular cracks at the base of lateral roots (Sprent, 2007). Once inside the nodule cells the rhizobia differentiate into plant-dependent bacteroids (Kereszt *et al.*, 2011). In this state, they produce nitrogenase, an enzyme capable of catalysing a break in the triple bond of gaseous nitrogen and transforming it into ammonia, a form available for assimilation by the plant (Downie, 2014). In return the plant provides the bacteria with a carbon source, in the form of dicarboxylic acids, and a sheltered environment (Kraiser *et al.*, 2011).

2.2 Agriculture and the exploitation of symbiotic nitrogen fixation

A consequence of root-nodule symbiosis is the replenishment of nitrogen in the surrounding soil, which acts as a natural fertilizer (Evans *et al.*, 2001). This allows for the use of legumes as rotational crops and for intercropping. This is particularly important in sparsely populated and resource-poor countries where fertilization is more difficult (Crews & Peoples, 2004). These practices re-nitrogenate the soil for subsequent and surrounding plants thereby decreasing the need for industrial fertilizers (Ofori & Stern, 1987; Peoples *et al.*, 2009). The use of legumes for intercropping, growing two or more crops in close proximity, and as rotational crops to enrich soil nitrogen has been dated back to ancient Greece ca. 300 BC (Papanastasis *et al.*, 2004). It has been reported that the Ancient Romans grew legumes and ploughed them under the soil (Heinrich, 2000). The industrial revolution brought with it an increase in agricultural production through the mass production of farming tools, effectively ending famine in many parts of the world which, in combination with medical advances and sanitation, assisted an increase in global population (Hounshell, 1985). This ever-increasing global population has been sustained due to the invention of industrial fertilizers, an advent reliant on the fixation of atmospheric nitrogen via the Haber-Bosch process (Brightling, 2018). Currently, over half of the global atmospheric nitrogen that is fixed yearly, 17.5×10^{10} kg (Hillel, 2007), comes from the production of industrial fertilizer. The creation and use of industrial fertilizer is energetically expensive and can be environmentally harmful due to a reliance on fossil fuels and the production of CO_2 (Bohlool *et al.*, 1992; Guo *et al.*, 2019). Symbiotic nitrogen fixation in legumes is estimated to fix between 8×10^{10} kg (Hillel, 2007) and 2.0×10^{10} kg (Herridge, 2008) per year. In recent times the collective conscience surrounding environmentally damaging practices has encouraged a switch to more sustainable farming practices. One such avenue for this would be exploiting root-nodule nitrogen-fixation as an alternative to industrial fertilizer. Future innovations in fertilizer technology are looking towards

utilising nitrogen fixing bacteria as a sustainable alternative to industrial methods (Bhattacharjee *et al.*, 2008). Whether that be genetically altering plants by introducing nitrogen fixing mechanisms into the plants themselves (Mus *et al.*, 2016), engineering nitrogen-fixing activity in bacteria that colonise cereals (Ryu *et al.*, 2020; Haskett *et al.*, 2022), or designing microbial inoculants to create a soil microbiome for optimal plant yield (Andrews *et al.*, 2003; Santos *et al.*, 2019). For the latter to become a viable option for legumes, the microbiota of the plant and the stability of any inoculant must first be comprehensively understood. The stability of an inoculum relies on the abiotic and biotic factors of the soil, therefore understanding these dynamics within the soil is vital (Griffiths *et al.*, 2008; Tkacz *et al.*, 2015). To achieve this level of comprehension we first need to understand not only the binary interaction between rhizobia and legumes, but also consider the broader plant and soil microbiota.

2.3 Specificity of root-nodule symbiosis

Root-nodule symbiosis is initiated after molecular communication between the two organisms is established, and the bacteria chemotactically move towards the plant root (Currier & Strobel, 1976). The molecules involved in the two-way signalling between the host plant and symbiotic bacteria vary across species. Nod factors, the signalling molecules produced by rhizobia, can differ in the length of the backbone, the size of the fatty acid chain as well as the decorations at either end of the molecule (Long, 1996). The specific variations of this molecule contribute to determining which potential host will be receptive and therefore compatible for infection (Lerouge *et al.*, 1990). This symbiont-specificity is typically dictated by the cluster of *nod* genes located either on a 'symbiotic island' in the bacterial chromosome or on a symbiotic plasmid (Ding & Hynes, 2009). This genomic orientation simplifies the potential for horizontal gene transfer, which has been hypothesised to be one of the factors that diversify legume-rhizobia symbiotic partnerships (Mergaert *et al.*, 1997; Bamba *et al.*, 2019).

Bacterial exopolysaccharides (EPS) are secreted biopolymers that are also relevant for symbiosis specificity (Wang *et al.*, 2012). The presence of particular EPS molecules can determine the successful establishment of rhizobial infection. *Bradyrhizobium japonicum* loses its symbiotic-compatibility with *Glycine soja*, but not with *Glycine max*, upon the mutation of the EPS synthesis gene *exoB* (Parniske *et al.*, 1994). *Sinorhizobium meliloti* Rm41 is incompatible with *Medicago truncatula*, but becomes an effective symbiotic nitrogen-fixer upon the introduction of a plasmid carrying a *exo* gene fragment from the effective symbiont, *S. meliloti* Rm1021 (Simsek *et al.*, 2007). This indicates that there is plant-specific selection of the infecting bacteria that depends on the presence or absence of certain EPS. There are also

other molecular interactions inside the plant that contribute to advantaging more beneficial symbiotic bacteria over others. Gibberellin is a phytohormone that increases nodule size and decreases nodule number, which correlates with a greater number of rhizobial progeny within the nodule. A larger population provides a competitive advantage in niche colonisation and resource acquisition upon nodule senescence. *G. max* expresses gibberellin 3-oxidases inside its nodules which converts a gibberellin precursor, GA_9, produced by the effective symbiont, *Bradyrhizobium diazoefficiens*, into the bioactive gibberellin GA_4, thus providing the beneficial bacteria with a competitive advantage (Nett *et al.*, 2022). Variant decorations on EPS molecules and Nod factors, the ability for nodulation genes to be transferred between bacteria as well as species-specific adaptations that provide a competitive advantage, all contribute to the specific compatibility seen between host and symbiont.

2.4 Promiscuity of legume-rhizobia symbiotic pairings

The molecular variations possible in root-nodule symbiosis allows for a high level of specificity between the plant and bacteria. Despite this, like many natural mutualistic relationships, the range of partners a legume can symbiotically cooperate with falls across a spectrum (Perret *et al.*, 2000; Zahran, 2001; Gwata *et al.*, 2004; Bascompte, 2009). Promiscuous plants can be infected by a broad range of bacteria, while the more stringent are only compatible with a few partners (van Rhijn & Vanderleyden, 1995; Bascompte, 2009). It has been observed that wild plants have more symbiotic partners than cultivated plants, hinting that domestication and the more stable environment that goes with it may select against promiscuity (Mutch & Young, 2004). This is seemingly in contrast to the assumption that globally successful domesticated crops would have to make use of bacterial symbionts from the native wild legumes, thus pressuring the selection of less selective plants (Mutch & Young, 2004). Whether plant promiscuity is beneficial is often conditional as being more promiscuous can also benefit an invasive plant by allowing it to exploit local bacteria as well as symbionts that have been co-introduced (Fourie-Basson, 2013; Ndlovu *et al.*, 2013). However, whether this invasive success is due to plant promiscuity or propagule pressure and agricultural practices remains unclear (Keet *et al.*, 2017). Promiscuity can also be detrimental to the success of a plant due to the variation of nitrogen fixation efficiency between a plant and effective symbionts (Collins *et al.*, 2002; Schumpp & Deakin, 2010). This variation in effectivity does not necessarily impact competitiveness when it comes to nodulation (Westhoek *et al.*, 2017; Bourion *et al.*, 2018). For example, both effective and ineffective strains of *S. meliloti* from the same region were able to nodulate *Medicago sativa* when in competition with one another (Amarger, 1981).

2.5 Ineffective nodules

The efficiency of nitrogen fixation can vary depending on the symbionts (Collins *et al.*, 2002; Liu *et al.*, 2020). Not only are some compatible symbionts less efficient at fixing nitrogen than others, but some bacteria are also capable of nodulating particular plants without fixing any nitrogen at all (Sachs & Simms, 2008). This results in a more parasitic symbiosis and the starvation of the plant. As the biogenesis of nitrogenase and the conversion of atmospheric nitrogen to ammonia are energetically expensive processes for the bacteria, not doing these allows them to save energy, providing them with a competitive advantage (Denison, 2000). Often termed 'cheaters', these bacteria typically induce white nodules, instead of the usual pink (Viands *et al.*, 1979). This is due to the lack of leghemoglobin, which is required to facilitate the low oxygen environment needed for nitrogenase to function whilst still maintaining oxygen flux for cellular respiration (Viands *et al.*, 1979; de Lajudie & Huguet, 1988; Ott *et al.*, 2005; Singh & Varma, 2017). The advantage the cheaters obtain through not fixing nitrogen is counteracted in the event the plant can exert sanctions on non-fixing bacteria or reward cooperating bacteria (Simms & Taylor, 2002).

It has been stated that a plant can sanction these cheater bacteria by moderating resource allocation to less-effective nodules or enforcing the early senescence of sub-optimal nodules (Banba *et al.*, 2001; Kiers *et al.*, 2003; Sachs & Simms, 2006; Serova *et al.*, 2018). There is little mechanistic knowledge about how host plant sanctions function (Masson-Boivin & Sachs, 2018). One suggested reason for the persistence of sub-optimal nodulators, despite plant sanctions, are that they coinfect with beneficial symbionts (Kiers & Denison, 2008). One such example is that of *Rhizobium leguminosarum* Norway, which was co-isolated from *Lotus corniculatus* nodules with the beneficial symbiont *Mesorhizobium norwegicum* 1022 (Kabdullayeva, 2019). *R. leguminosarum* Norway cannot nodulate *L. corniculatus* alone and forms ineffective nodules on *Lotus burttii* (Gossmann et al., 2012). Although, it has also been shown that some legumes can detect and even sanction nodules with a mix of effective and ineffective bacteria (Regus *et al.*, 2017). Another explanation is that they are just mismatched and do in fact effectively fix nitrogen in other plant species (Kiers & Denison, 2008). *Rhizobium etli* can nodulate and infect *Lotus japonicus* but fixes nitrogen at a sub-optimal level (Banba *et al.*, 2001). After 3 weeks, fixation suddenly halts, and the nodules degrade. However, other sub-optimal *Rhizobium* symbionts are able to sustain an infection in *L. japonicus* for several months indicating that efficiency of nitrogen fixation is not necessarily required for persistence in nodules (Schumpp *et al.*, 2009; Schumpp & Deakin, 2010). The persistence of cheating nodulators despite plant sanctions and competition from beneficial symbionts begs a closer look at the external root-nodule environment and the potential impact of microbes therein.

3. The soil microbiota and root-nodule symbiosis

3.1 Abiotic and biotic factors that influence the soil microbiota

Soils typically harbour a diverse range of microbes that vary dependent many abiotic parameters such as carbon:nitrogen ratio, pH, soil grain size, temperature, tillage, moisture, and depth to name a few (Zarraonaindia *et al.*, 2015; Adamczyk *et al.*, 2019; Sharaf *et al.*, 2019; Kraut-Cohen *et al.*, 2020; Wang, S *et al.*, 2021; Sokol *et al.*, 2022). Soil with a neutral pH typically has a higher microbial diversity than those that are acidic or alkaline, due to the effect pH has on nutrient availability and microbial enzymatic activity (Cao *et al.*, 2016; Wu *et al.*, 2017; Custódio *et al.*, 2022). Many abiotic factors are interconnected and therefore have an indirect effect on the soil microbiome. For example, grain size heterogeneity correlates with microbiome variation in the permeable sediment of the Qi river in China. Heterogeneity of grain size confers a change in sediment chemistry, which in turn has a significant effect on the microbiome (Wang, S *et al.*, 2021). Similarly, tillage of soils reduces the hydrolytic and redox activity of the microbiota present, which results in a divergent microbiome to that seen in soils without tillage (Kraut-Cohen *et al.*, 2020).

Biotic factors that influence the soil microbiome stem largely from fauna, microbes and plants (Zhu *et al.*, 2020; Sokol *et al.*, 2022). Predation of soil bacteria by protists, nematodes and microarthropods has a substantial influence on the overall microbial makeup of the soil (Bonkowski, 2004; Thakur & Geisen, 2019). Similarly, there are indirect effects from other soil microorganisms that shape the structure of the microbiome, largely via competition for nutrients and supplying nutrients via decomposition (Thakur & Geisen, 2019).

3.2 Plant influence on the soil microbiota

The proximity to a plant and the developmental stage of the plant also influences the soil microbial community. This is done via exudates that the plant root secretes into the soil (Knudson, 1920; Badri & Vivanco, 2009; Sasse *et al.*, 2018). These exudates can encourage the recruitment of specific bacteria towards or away from the plant root system (Currier & Strobel, 1976; el Zahar Haichar *et al.*, 2014). This acts as a natural filter so that each region closer to the root has a less diverse microbial community as root exudates change soil conditions to favour some bacteria over others (Huang *et al.*, 2020; Munoz-Ucros *et al.*, 2021). Due to this the bulk soil, soil outside the rhizosphere, has the most diverse but least plant-influenced microbial community as it is further away from the plant and only exposed to a dissipating level of root exudate (Hartman & Tringe, 2019; Huang *et al.*, 2020). The soil directly surrounding the root is not only susceptible to influence by plant exudates, but also physically

due to root configuration. Dense root architecture can encourage the aggregation of nutrients and moisture thereby facilitating the distinction of micro-environments that are more suited for select microbes (Ho *et al.*, 2005; Saleem *et al.*, 2018). An even more selective niche is that of the rhizoplane, comprised of rhizosphere bacteria that can adhere to the root epidermal cells (Chave *et al.*, 2008). Lastly, microbes that can endophytically colonise the plant are a distinct assemblage of rhizosphere bacteria that are able to infiltrate the plant system (Hallmann & Berg, 2006; Gottel *et al.*, 2011). This natural selectivity imposed by the plant on soil microbiota has pressured the evolution of microbes specialised for these niches (Badri & Vivanco, 2009). For example, there are a higher percentage of microbes that require exogenous plant-derived amino acids for optimal growth in the rhizosphere than there are in the bulk soil, and an even higher percentage in the rhizoplane (Ramey *et al.*, 2004; de Ridder-Duine *et al.*, 2005).

A plant can exert a more specific influence over the microbiota directly surrounding the root compared to the rhizosphere. For instance, the roots of *Triticum aestivum* (wheat), *Zea mays* (maize), *Brassica napus* (rape) and *M. truncatula* (barrel clover) are colonised by different bacterial communities due to their differences in plant exudate profile, however this distinction was less pronounced in the rhizosphere (Haichar *et al.*, 2008). Plants can exert selective pressure on the rhizo-compartments that bacteria inhabit. *L. japonicus* mutants incapable of establishing root-nodule symbiosis, have an altered root community. However, this was based on plant genetic factors, rather than an indirect effect due to the lack of nitrogen fixation, indicating that the plant directly influences the selection of root bacteria (Zgadzaj *et al.*, 2016).

Plant microbial communities are also dynamic (Zhalnina *et al.*, 2018). The rhizoplane and rhizosphere contain microbiota that vary temporally, with the mature parts of the root having a distinct microbiome to those more nascent (Massalha *et al.*, 2017; Munoz-Ucros *et al.*, 2021). This is likely due to the composition of root-exudates differing along the length of the root as it develops which chemotactically recruit specific bacteria at different times and at different root sections (Aulakh *et al.*, 2001; Massalha *et al.*, 2017; Zhalnina *et al.*, 2018; Edmonds *et al.*, 2020). *Arabidopsis thaliana* and *T. aestivum* can select for a subset of microbes at certain stages of development, supposedly to aid in apposite plant functions (Chaparro *et al.*, 2014; Chen, S *et al.*, 2019). While plant can chemically encourage the formation of a particular microbial community in the soil via exudates, soil microbiota can conversely also influence the microbiota of the plant. For instance, the phyllosphere of a grapevine has more bacterial strains in common with its own rhizosphere than with the phyllosphere of other plants in the same region (Zarraonaindia *et al.*, 2015). This suggests that the soil acts as the main microbial reservoir for the rest of the plant microbiota, although some bacteria can also originate from the seed and the air (Sánchez-Cañizares *et al.*, 2017).

All the factors that influence the microbiota around a plant have forced the evolution of bacteria that are not only suited to the niche, but also some that are synergistic with the plant (Zilber-Rosenberg & Rosenberg, 2008; Lugtenberg & Kamilova, 2009).

3.3 Plant-beneficial bacteria

Plant synergistic bacteria that inhabit soil benefit the host plant in both indirect and direct ways (Verma *et al.*, 2010). Plant-growth-promoting bacteria (PGPB) can indirectly benefit the plant by secreting antimicrobial compounds or siderophores with a higher affinity for soil iron than phytopathogenic competitors (Glick, 2012; Shobha & Kumudini, 2012; Chen, L *et al.*, 2019). Other benefits of PGPB act directly on the plant. Direct benefits of PGPB can be through the production of phytohormones, such as cytokinins and auxins (Liu *et al.*, 2013; Duca *et al.*, 2014). Indoleacetic acid is an auxin that can aid in root elongation, plant cell division and lateral root formation (Khalid *et al.*, 2004; Schlicht *et al.*, 2013; Duca *et al.*, 2014). Another benefit PGPB confer is in assisting the plant in resource acquisition, especially of nutrients which can exist in states of low bioavailability, such as insoluble phosphate and atmospheric nitrogen (Khan *et al.*, 2009; Dawwam *et al.*, 2013; De Bruijn, 2015). Root-nodule symbiosis is one such example of bacteria assisting the plant by making unreactive atmospheric nitrogen available, a process that is critically important in low-nitrogen soils. Nitrogen-fixing bacteria are the most researched PGPB inside legume nodules, although evidence suggests that there may be other beneficial microbes that contribute to this symbiosis as well (Martínez-Hidalgo & Hirsch, 2017).

4. The nodule microbiome

4.1 Soil influence on the nodule microbiome

The nodule microbiota can vary dependent on a range of factors. The reservoir from which most nodule bacteria are selected is in the soil, therefore variation to the soil microbiota can subsequently affect that of the nodule (Sánchez-Cañizares *et al.*, 2017; Mukhtar *et al.*, 2020). The soil conditions and inhabiting plants can influence the proliferation of the native bacteria (Brant *et al.*, 2006; Berg & Smalla, 2009; Zarraonaindia *et al.*, 2015; Wang, W *et al.*, 2021). Certain conditions will suit the proliferation of particular bacteria, giving them a competitive advantage in expanding into new niches (Bai *et al.*, 2019; Windels *et al.*, 2020). For example, which rhizobial species colonise *Sesbania cannabina* nodules depends on pH and soil salinity, with *Bradyrhizobium* spp. and *Sinorhizobium* spp. more favoured in neutral and saline-alkali soil, respectively (Zhang *et al.*, 2011; Li *et al.*, 2016). This variation in soil conditions can be environmentally driven or due to human intervention. Agricultural practices, such as irrigation, can alter the soil microbiome and subsequently the nodule microbiome (Sharaf *et al.*, 2019).

4.2 Plant host influence on nodule microbiota

Nodulation phenotype can vary across plant genotypes (Gossmann *et al.*, 2012), however whether this translates into a significant variation in nodule microbiota is unclear. The influence of plant genotype on microbial assembly is difficult to parse, due to it being intertwined with the environmental conditions and microbiota therein. It has been suggested that plant-microbe coevolution facilitated by cross-kingdom signalling has meant plants generally adapt a holobiont related to the environment (Vandenkoornhuyse *et al.*, 2015). For example, several genotypes of *Vigna unguiculata* from different regions of Africa are colonised by a diverse range of *Bradyrhizobium* symbionts (Pule-Meulenberg *et al.*, 2010). Whether this facilitated a variation in the broader microbiome is unclear. A similar study of different *V. unguicalata* genotypes in Brazil showed that non-rhizobial nodule endophyte differences were mainly due to soil influence, rather than host genotype (Leite *et al.*, 2017). Further ambiguity about the influence of genotype is seen when considering the microbiota in other plant compartments. Root endophyte diversity of *M. truncatula* can vary with genotype, although this same microbial variation was not seen in the nodule (Brown *et al.*, 2020). However, using other metrics to track differences in nodule endophytes between *M. truncatula* genotypes revealed that there was in fact some variation, not in the number of different species (diversity), but rather in the particular strains present (composition) (Burns *et al.*, 2021).

The microbiota of cultivated and wild plant nodules can vary. Wild *Sulla coronaria* nodules have more culturable non-rhizobial endophytes than those of cropped plants (Muresu *et al.*, 2019). It was subsequently hypothesised that these other endophytes play a role in alleviating the negative effects of the harsher environmental conditions in the wild. Contrastingly, the cultivated *G. max* has a higher number of non-rhizobial endophytes in nodules than the closely related wild *G. soja* (Zheng *et al.*, 2020). The hypothesis is that these differences stem from the ability of each plant to adapt to salt-alkali stress conditions. For both of these examples, in terms of recruiting bacteria to the nodule, the cultivation of the plant is less relevant than the conditions to which the plant is subjected. However, other observations in *G. max* have shown variation in nodule community between cultivars grown under the same conditions (Sharaf *et al.*, 2019). This indicates that the variation between cultivated and wild plant nodules may be influenced by more than just the environmental conditions of each niche.

4.3 Plant growth promoting bacteria in nodules

Various bacterial species that do not induce nodules alone have been found to not only co-colonise plant nodules but also contribute to the health of the plant (Martínez-Hidalgo & Hirsch,

2017). A broad-range of other *Alpha*-, *Beta*- and *Gammaproteobacteria*, as well as *Firmicutes* and *Actinobacteria* have been isolated from a range of legume species (Martínez-Hidalgo & Hirsch, 2017). The fixation of atmospheric nitrogen is the plant growth-promoting trait common to host-compatible rhizobia, while other nodule-colonising bacteria have shown alternative plant growth-promoting traits. For instance, several *Pseudomonas* spp. can manipulate the plant hormone homeostasis through the direct production of plant hormones like indoleacetic acid (Duca *et al.*, 2014; Gopalakrishnan *et al.*, 2015; Kumawat *et al.*, 2019). They can also produce enzymes involved in the biosynthesis of hormones, such as the 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, which can convert ACC, the precursor of ethylene, into ammonia and α-ketobutyrate, thereby lessening the stress-induced plant growth suppression that ethylene can cause (Kong *et al.*, 2017; Kumawat *et al.*, 2019). They can also suppress antagonistic bacteria by outcompeting them for iron using siderophores they produce or secreting antimicrobial compounds (Sindhu & Dadarwal, 2001; Dey *et al.*, 2004; Zhao *et al.*, 2013; Chen, L *et al.*, 2019). PGPB can assist the plant in nutrient acquisition by solubilising phosphate (Argaw, 2012; Zhao *et al.*, 2013) or also aid in coping with environmental stresses caused by metal ions or salinity (Egamberdieva *et al.*, 2013; Kong *et al.*, 2017; Xiong *et al.*, 2020). Some nodule colonisers can effectively form biofilms to withstand various biotic and abiotic stresses, potentially contributing to the successful root colonisation of symbiotic bacteria (Tariq *et al.*, 2014). *Agrobacterium tumefaciens* can form dense, complex biofilms with rhizobia, like those that rhizobia form on the root epidermis and root hairs, which potentially serve as the reservoir for infection (Ramey *et al.*, 2004). *A. tumefaciens* can also inhabit nodules, however whether they infect via the same route as rhizobia is unclear (Wang *et al.*, 2006).

4.4 Non-rhizobial bacteria and nodule colonisation

The natural filtration that occurs from the bulk soil to the rhizosphere to colonising the root already limits microbial diversity (Huang *et al.*, 2020; Munoz-Ucros *et al.*, 2021). The barrier to then becoming nodule-colonising also requires entering the plant and then navigating the internal conditions (Compant *et al.*, 2021). Symbiotic rhizobia do this via *nod* genes that facilitate specific two-way signalling recognition and cooperation with the plant (Debellé *et al.*, 2001). Non-rhizobial nodule colonisers lack these genes for this signalling process and yet still infect the nodule (Pandya *et al.*, 2013; Zgadzaj *et al.*, 2015). Whether the mode of colonisation is via cracks in the root epidermis or via infection threads is unclear. However, the lack of compatible *nod* genes does not necessarily indicate that non-rhizobial nodule colonisers do not play role in the infection process as there are other molecular bacterial components, such as EPS, can also be important for a successful symbiotic pairing (Jones *et*

al., 2008). An example of such a bacterium that co-colonises nodules is *Rhizobium mesosinicum* KAW12. This strain lacks *nod* and *nif* gene clusters that encode proteins essential for nodulation and nitrogen fixation, respectively. KAW12 cannot nodulate *L. japonicus* by itself, however it is a competent coloniser of *L. japonicus* when co-inoculated with the symbiont *Mesorhizobium loti* R7A. An *exoU* EPS mutant of *M. loti*, which rarely produces infected nodules, could have the infection process restored upon co-inoculation with KAW12, suggesting that the EPS of KAW12 was able to complement the function lost in the *M. loti exoU* mutant (Zgadzaj *et al.*, 2015). This shows that although this non-symbiont could not nodulate by itself, it did have some molecular characteristics that can contribute to nodulation. Whether this is the case for other nodule endophytes remains unclear.

4.5 Current state of research

The benefit PGPB provide plants is empirically exhibited upon their co-inoculation with rhizobia. Plant growth promotion due to non-rhizobial bacteria is seen in *G. max* (Kumawat *et al.*, 2019), *Medicago lupulina* (Kong *et al.*, 2017), *Cicer artietinum* (Malik & Sindhu, 2011; Gopalakrishnan *et al.*, 2015), *Saphora alopecuroides* (Zhao *et al*., 2013), *Galega orientalis* Lam. (Egamberdieva *et al.*, 2010), and *Arachis hypogea* L. (Dey *et al.*, 2004) to name a few. Most of these studies show a correlation between the presence of plant growth-promoting activities and plant growth improvement after co-inoculation, typically increased plant yield or nodulation, without considering the broader microbiome that is naturally present. The myriad potential microbe-microbe interactions possible in a soil environment could have indirect effects on both rhizobia and non-rhizobia thereby compromising any potential plant benefit they might provide. Research analysing host-symbiont specificity and ineffective nodules has been done with binary systems, ignoring any potential effect from the broader plant and soil microbiota. With the advent of improved deep sequencing technologies, microbiome characterisation has become a practical way of analysing the microbial makeup of particular niches. Comprehensive nodule microbiome studies are becoming more prevalent and can provide insights into the potential interactions that could shape the function of PGPB (Hansen *et al.*, 2020; Rocha *et al.*, 2020; Zheng *et al.*, 2020).

Aims of thesis

The soil is one of the most complex bacterial niches that exists in the natural world. The potential number of microbe-microbe interactions and the possible level of interconnectedness that circulates in the soil is almost unfathomable, therefore we find it remiss not to consider its potential importance in root-nodule symbiosis. We hypothesised that other non-symbiotic soil bacteria may also play a role in the functionality of root-nodule symbiosis. The massive complexity of plant-associated microbes and the compartments they inhabit, made practically addressing this hypothesis difficult. We therefore reduced the scope of our attention to only include nodule endophytes.

It is well established that non-rhizobial bacteria can also colonise nodules, however plant response to this colonisation is not well understood. To that end our first aim was to **assess how and if the nodule microbiome of *Lotus* varies in differing soil inoculants, genotypes and plant health phenotypes.** To do this we used gnotobiotically germinated plants inoculated with two different soil suspensions in closed-pot system to minimise abiotic influences in order to distinguish microbe-driven variation between the sample types. Next generation sequencing of the microbiome of surface-sterilised nodules allowed us to fully characterise all bacteria that endophytically colonised the different sample types. Assigning taxonomy to reads clustered as amplicon sequence variants (ASVs) then allowed us to observe typical colonisation patterns of the particular sample types.

Our next aim was to focus on **which ASVs were characteristic of differences between the sample types**, in a bid to unveil potentially important strains. Comparative analyses using support vector machine learning and a network algorithm were used to uncover potential interactions and pivotal ASVs.

The next aim was to qualify **what were the potential roles and nodule colonisation pattern of nodule-isolated strains representative of the ASVs-of-interest**. This was done by aligning said ASVs with strains isolated from nodules of *Lotus* that had been inoculated with the soil suspensions. Information garnered from the bioinformatic analyses then informed the creation of synthetic inoculations in a bid to recreate observed phenotypes and pin-point the role of particular nodule endophytes. The plants treated with the synthetic inoculations were then observed for phenotypic variation. In parallel to this we used confocal microscopy to image the localisation inside the nodule of fluorescently tagged strains in a bid to better understand the putative role that they play in root-nodule symbiosis and confirm their endophytic presence in nodules.

A parallel aim of this project was to **characterise the newly described *M. norwegicum*,** a rhizobia co-isolated from *Lotus corniculatus* with the *Lotus*-parasitic rhizobia, *R. leguminosarum* Norway.

Results

Publication I:

Mesorhizobium norvegicum sp. nov., a rhizobium isolated from a *Lotus corniculatus* root nodule in Norway

INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY

TAXONOMIC DESCRIPTION

Kabdullayeva *et al.*, *Int. J. Syst. Evol. Microbiol.* 2020;70:388–396

DOI 10.1099/ijsem.0.003769

MICROBIOLOGY SOCIETY

Mesorhizobium norvegicum sp. nov., a rhizobium isolated from a *Lotus corniculatus* root nodule in Norway

Tolganay Kabdullayeva, Duncan B. Crosbie and Macarena Marín*

Abstract

Strain 10.2.2^T was isolated from a root nodule of a *Lotus corniculatus* plant growing near Skammestein (Norway). Phenotypic and chemotaxonomic characterization revealed that colonies grown on yeast–mannitol broth agar were circular, convex and slimy. Growth occurred at 28 °C in 0–1 % NaCl and in a pH range from above 4 to 10. Cells were resistant to kanamycin and phosphomycin. They could assimilate carbon sources such as L-lysine, D-mannose, D-mannitol, and L-alanine. Major fatty acids found in the organism were 11-methyl $C_{18:1}\omega7c$, $C_{16:0}$, $C_{18:1}\omega7c$, $C_{18:0}$ and $C_{19:0}$ cyclo $\omega8c$. Genome sequencing and characterization of the genome revealed its size to be 8.27 Mbp with a G+C content of 62.4 mol%. Phylogenetic analyses based on the 16S rRNA gene and housekeeping gene alignments placed this strain within the genus *Mesorhizobium*. Pairwise genome-wide average nucleotide identity values supported that strain 10.2.2^T represents a new species, for which we propose the name *Mesorhizobium norvegicum* sp. nov. with the type strain 10.2.2^T (=DSM 108834^T=LMG 31153^T).

The legume–rhizobia symbiosis plays a crucial role in the global nitrogen cycle, as it is a major source of biological nitrogen fixation in terrestrial ecosystems [1, 2]. In agriculture, biological nitrogen fixation has been proposed for a long time as a sustainable alternative to synthetic nitrogen fertilizers [3–5]. However, it has recently gained more attention under the scope of the United Nations Sustainable Development Goals [6]. The exploration of rhizobial diversity might help us improve sustainability in agriculture by identifying novel rhizobia strains with higher nitrogen-fixation capacities.

Rhizobia are a paraphyletic group of diazotrophic bacteria that belong to at least three genera of betaproteobacteria and 17 genera of alphaproteobacteria [7]. Among these, the genus *Mesorhizobium* is one of the best investigated in terms of diversity with 51 species described to date (www.bacterio.net/mesorhizobium.html). The genus was established formally by Jarvis *et al.* in 1997 [8] and comprises Gram-negative, non-sporulating, rod-shaped, motile, chemoorganotrophic rhizobacteria [9]. *Mesorhizobium* species have an intermediate growth rate in comparison to fast-growing *Rhizobium* and slow-growing *Bradyrhizobium* species [7] and this is where this genus received its name [8, 10].

The host specificity of *Mesorhizobium* expands across different legume genera including *Acacia*, *Cicer*, *Prosopis*, *Sophora* and *Lotus* [11]. Among these, *Lotus* plants, such as *Lotus corniculatus*, are a rich reservoir of *Mesorhizobium* strains. *L. corniculatus*, commonly known as bird's-foot trefoil, is a geographically widely distributed flowering perennial grass that is native to Scandinavian wetlands, temporal Eurasian meadows and North African grasslands [12]. Some of the type strains isolated from this species are *Mesorhizobium loti* [13], *Mesorhizobium helmanticense* [14] and *Mesorhizobium olivaresii* [15].

Here we describe strain 10.2.2^T, which was isolated from a *L. corniculatus* root nodule growing in a field near Skammestein in Norway [16]. Based on the phylogeny of the 16S rDNA and three housekeeping genes we placed this strain within the genus *Mesorhizobium*. Moreover, the results of polyphasic analysis supported that this strain belongs to a novel bacterial species. We propose to establish for strain 10.2.2^T the species name *Mesorhizobium norvegicum* sp. nov.

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Keywords: *Mesorhizobium*; *Lotus corniculatus*; root nodule symbiosis; rhizobia; genome; average nucleotide identity.
Abbreviations: ANI, average nucleotide identity; MLSA, multilocus sequence analysis; YMB, yeast–mannitol broth.
The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and the genome sequences are MK908404.1 and SMYZ00000000 for strain 10.2.2 and MK908405.1 and SMYY00000000 for 10.2.3, respectively.
Three supplementary tables are available with the online version of this article.

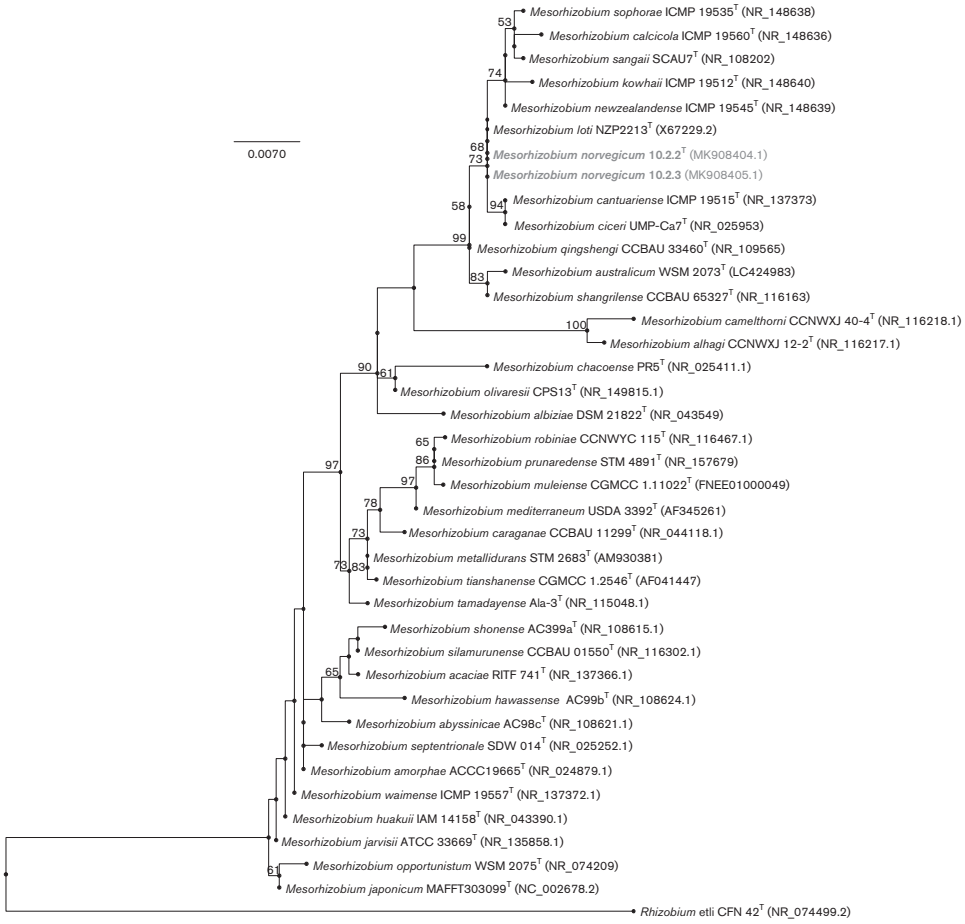

Fig. 1. Maximum-likelihood phylogenetic tree based on 16S rRNA gene sequences (1200 bp) showing the position of isolates 10.2.2^T and 10.2.3 within the genus *Mesorhizobium*. Bootstrap values calculated for 1000 replications are indicated (shown only when ≥70%). Accession numbers are shown in parentheses. *Rhizobium etli* CFN42^T was used as an outgroup.

ISOLATION AND ECOLOGY

In a previous study, strain 10.2.2^T was isolated from a root nodule collected from a *L. corniculatus* plant growing in Skammestein, Norway. The geographic coordinates of the collection site are latitude 61° 10′ 54.6″ and longitude 08° 57′ 54.5″ [16]. The strain was isolated from a nodule after dissection and surface-sterilization in a 10% bleach and 0.1% sodium dodecyl sulfate solution. The content of the crushed nodule was serially diluted in 20Q liquid medium [17], and the dilutions were plated on 20Q agar plates. Three isolates were purified from that nodule. Based on 16S rRNA gene phylogeny, two of them, 10.2.2^T and 10.2.3, belonged to the genus *Mesorhizobium*, whereas the third isolate was a *Rhizobium leguminosarum* [16, 18]. Interestingly, isolates 10.2.2^T and 10.2.3 clustered separately from other described *Mesorhizobium* strains [16].

GENOME FEATURES

To characterize these isolates, the genomes of 10.2.2^T and the co-isolated 10.2.3 were sequenced and annotated. Bacteria were grown at 28 °C and 180 r.p.m. for 2 days in tryptone–yeast

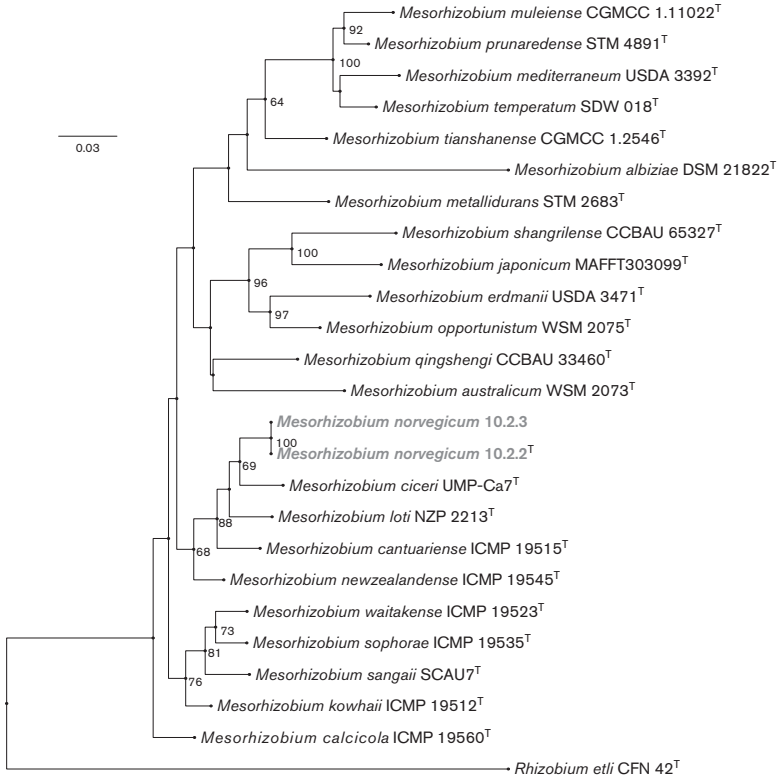

Fig. 2. Maximum-likelihood phylogenetic tree based on concatenated gene sequences of *glnII* (522 bp), *rpoB* (412 bp) and *recA* (328 bp) fragments showing the position of isolates 10.2.2^T and 10.2.3. Bootstrap values calculated for 1000 replications are indicated (shown only when ≥70%). *Rhizobium etli* CFN42^T was used as an outgroup.

broth. Genomic DNA was isolated from 30 ml of the respective bacterial suspensions (OD_{600}=1.0) using the CTAB method [19]. The quality of the isolated DNA samples was determined by gel electrophoresis and NanoDrop spectrophotometry. Library preparation and whole-genome sequencing were conducted at the Genomics Service Unit (LMU Biocenter, Munich). Briefly, genomic DNA libraries were prepared with the Nextera Kit (Illumina) following the manufacturer's protocol. Short-read paired-end sequencing (2×150 bp, v2 chemistry) was performed using an Illumina MiSeq sequencer yielding 1079958 and 1316698 paired reads and 1.5 Gb of primary sequences for 10.2.2^T and 10.2.3, respectively.

Quality control and absence of contamination in the sequenced reads was evaluated using the CLC Genomics Workbench 9.0.1 (QIAGEN) and the ContEst16S online tool [20]. The sequenced reads were assembled *de novo* into contigs by using NGS Core Tools in CLC Genomics Workbench 11.0.1 and the quality of the assemblies was assessed using the Quality Assessment Tool for Genome Assemblies within the webtool QUAST (http://quast.bioinf.spbau.ru/) [21]. The contig assemblies were annotated automatically using the MicroScope platform [22–24]. The characteristics of the genome assemblies as generated by MicroScope are summarized in Table S1 (available in the online version of this article). The GenBank accession numbers for the genome assemblies of strains 10.2.2^T and 10.2.3 are SMYZ00000000 and SMYY00000000, respectively.

The draft genomes of 10.2.2^T and 10.2.3 were highly similar. They consisted of 8.27 Mbp, had 62.4mol% G+C content, and contained 54 and 50 RNA genes, respectively (Table S1). These are comparable to values extracted from the published genomes of other *Mesorhizobium* type strains (Table S2). A BUSCO analysis [25, 26] of the draft genomes of both isolates revealed 97.6 and 98.4 % completeness of the expected

Table 1. Calculated OrthoANI values based on whole-genome comparisons between Mesorhizobium *norvegicum* isolates and *Mesorhizobium* type strains

Mesorhizobium cantuariense ICMP 19515^{T} and *Mesorhizobium newzealandense* ICMP 19545^{T} were not included in the analysis as no genome data was available.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. *Mesorhizobium norvegicum* 10.2.2^{T}	100																
2. *Mesorhizobium norvegicum* 10.2.3	99.9	100															
3. *Mesorhizobium loti* NZP 2213T	93.6	93.5	100														
4. *Mesorhizobium ciceri* UMP-Ca7^{T}	91.1	91.1	90.9	100													
5. *Mesorhizobium sophorae* ICMP 19535^{T}	89.0	89.0	88.8	88.1	100												
6. *Mesorhizobium qingshengii* CCBAU 33460^{T}	88.6	88.7	88.6	87.7	88.1	100											
7. *Mesorhizobium australicum* WSM 2073^{T}	86.1	86.1	85.9	85.7	85.6	86.1	100										
8. *Mesorhizobium tianshanense* CGMCC 1.2546^{T}	84.3	84.4	84.1	83.9	84.2	84.4	83.4	100									
9. *Mesorhizobium temperatum* SDW 018^{T}	84.5	84.5	84.1	83.9	84.2	84.3	83.2	91.1	100								
10. *Mesorhizobium mediterraneum* USDA 3392^{T}	84.6	84.6	84.3	84.7	84.2	84.3	83.2	90.8	92.2	100							
11. *Mesorhizobium pranuredense* STM 4891^{T}	84.3	84.3	83.9	83.9	83.9	84.3	83.4	89.9	92.9	91.3	100						
12. *Mesorhizobium mulense* CGMCC 1.11022^{T}	84.3	84.4	83.9	84.6	83.9	84.2	83.1	89.8	92.8	91.9	94.8	100					
13. *Mesorhizobium metallidurans* STM 2683^{T}	85.1	85.2	84.9	84.5	84.9	85.1	84.1	87.1	87.1	87.3	87.4	86.6	100				
14. *Mesorhizobium opportunistum* WSM 2075^{T}	87.3	87.1	87.3	86.9	86.6	87.1	88.5	83.6	83.6	83.9	83.6	83.5	84.8	100			
15. *Mesorhizobium ehimanii* USDA 3471^{T}	87.2	87.2	87.1	86.1	86.0	86.5	86.9	83.5	83.5	83.3	83.3	83.3	84.2	87.7	100		

Continued

Table 1. Continued

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
16, *Mesorhizobium japonicum* MAFF303099^{T}	87.8	87.9	87.4	86.5	86.6	87.0	87.2	83.9	83.7	83.8	83.6	83.7	84.4	88.3	89.8	100	
17, *Mesorhizobium albiziae* DSM 21822^{T}	77.5	77.6	77.6	77.5	77.6	77.5	77.4	78.0	78.0	77.6	77.8	77.8	78.1	77.5	77.6	77.6	100

gene set for *Rhizobiales*, respectively. Synteny analysis using MicroScope revealed that 95.91% of the predicted CDS form syntheny groups. Thus, these isolates most likely belong to the same strain.

A comparison of the draft genomes of 10.2.2^{T} and 10.2.3 to the genomes of other *Mesorhizobium* strains using MicroCyc in MicroScope did not reveal major metabolic differences. Both draft genomes contained symbiotic genes, such as the *nod* and *nol* genes involved in the synthesis and export of the nodulation factor, and the *nif* and *fix* genes required for nitrogen fixation. The organization of these genes was syntenic to orthologs in other type strains, such as *Mesorhizobium japonicum* MAFF303099^{TT} [27].

PHYLOGENY

The 16S rRNA gene is not a reliable phylogenetic marker for the genus *Mesorhizobium*, as several species contain identical 16S rRNA gene sequences, but have other divergent housekeeping genes [28]. Thus, to determine the phylogenetic placement of strain 10.2.2^{T}, we not only reconstructed a phylogenetic tree of the 16S rRNA gene, but also trees of concatenated sequences of the *glnII*, *rpoB* and *recA* housekeeping genes. Sequences were extracted from the annotated draft genomes of isolates 10.2.2^{T} and 10.2.3 and aligned with those of *Mesorhizobium* type strains publicly available at the NCBI database. Accession numbers of retrieved sequences can be found in Table S3. Alignments of the individual gene sequences were performed using MAFFT version 7 [29]. After trimming, the housekeeping gene sequences were concatenated manually. The resulting alignments were used to reconstruct phylogenetic trees by using the maximum-likelihood method using RAxML-HPC BlackBox [30] in the CIPRES Science Gateway version 3.3 platform [31]. Bootstrap values were calculated for 1000 iterations.

Pairwise sequence comparisons of a 1200 bp long 16S rRNA gene fragment of 50 *Mesorhizobium* type strains revealed that the 16S rRNA gene of strain 10.2.2^{T} was identical to the sequence of isolate 10.2.3 and of *M. loti* NZP2213^{T}. However, it shared >99.5% sequence similarity with an additional 10 type strains. Based on this, we selected non-redundant sequences (1270 bp long) of 36 *Mesorhizobium* species, including all closely related strains, to reconstruct the 16S rRNA gene phylogeny. Isolates 10.2.2^{T} and 10.2.3 clustered together in a clade within the *Mesorhizobium* genus (Fig. 1). To resolve the phylogenetic relationship within this group we conducted multiple locus sequence analysis (MLSA) with concatenated sequences of fragments of three housekeeping genes: *glnII* (522 bp), *rpoB* (412 bp) and *recA* (328 bp). Isolates 10.2.2^{T} and 10.2.3 formed a separated group within a branch containing *M. ciceri* UMP-Ca7^{T}, *M. loti* NZP 2213^{T}, *M. cantuariense* ICMP 19515^{T} and *M. newzealandense* ICMP 19545^{T} (Fig. 2). Moreover, pair-wise comparisons of these MLSA sequences revealed that these strains share less than 97% identity with 10.2.2^{T} and 10.2.3. This suggests that isolates 10.2.2^{T} and 10.2.3 belong to a novel species of the genus *Mesorhizobium*.

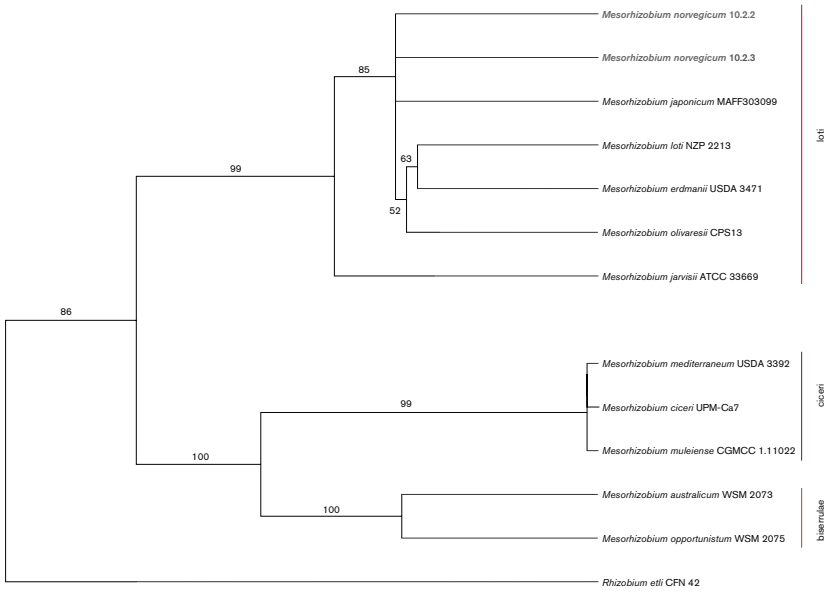

Fig. 3. Maximum-likelihood phylogenetic tree based on NodC protein sequences of isolates 10.2.2^T and 10.2.3 and 18 members of the *loti* symbiovar. Bootstrap values calculated for 1000 replications are indicated. *Rhizobium etli* CFN42^T was used as an outgroup.

To further validate the new species delineation, we conducted whole-genome comparisons and calculated the average nucleotide identity (ANI). We applied OAT 0.93.1, which uses orthologous regions in a pairwise comparison, to calculate OrthoANI values [32]. Strains are considered to belong to different species if they share less than 95–96% genome identity [32, 33]. The whole-genome comparison of isolates 10.2.2^T and 10.2.3 revealed 99.98% identity between them, which indicates that they belong to one species (Table 1). In contrast, the OrthoANI values of whole-genome comparisons between these isolates and 15 *Mesorhizobium* type strains were below the 95% cut-off threshold, supporting the separation of 10.2.2^T and 10.2.3 into a distinct species (Table 1).

SYMBIOTAXONOMY

Rhizobia are grouped in different symbiotic variants or symbiovars based on their symbiotic activity in the host plants and the sequence of certain symbiosis genes [34]. Symbiovars are believed to reflect the symbiont adaptation to the host plant [35]. Most *Mesorhizobium* strains isolated from *L. corniculatus* and other related *Lotus* species, including the type strains of *M. loti* and *M. japonicum*, are grouped in the symbiovar *loti* [36].

To validate that strain 10.2.2^T, which was isolated from *L. corniculatus*, also belongs to this symbiovar we performed a phylogenetic analysis based on NodC protein sequences. The analysis of the 170 amino acid long alignment was conducted with the MEGA X software [37] by using the maximum-likelihood method supported by 1000 bootstrap iterations. Isolates 10.2.2^T and 10.2.3 clustered together with other *Lotus* symbiont type strains, such as *M. japonicum* MAFF303099^T and *M. helmaticense* CSLC115N^T (Fig. 3). This suggests that isolates 10.2.2^T and 10.2.3 indeed belong to the *loti* symbiovar. Moreover, pairwise comparisons of the full NodC protein sequences performed with the Sequence Manipulation Suite [38] revealed 96.69% identity to the protein sequence from *M. japonicum* MAFF303099^T. Strain 10.2.2^T can effectively nodulate *L. corniculatus*, *L. japonicus*, *L. filicaulis*, *L. glaber* and *L. burttii*. In addition, it induces nodule primordia that do not fully develop in *L. pedunculatus* [16], a host that belongs to a different cross-inoculation group.

PHYSIOLOGY AND CHEMOTAXONOMY

The physiology and chemotaxonomy analyses were conducted with isolates 10.2.2^T and 10.2.3. Both isolates formed circular, convex, slimy colonies of moderate size after 5 days at 28 °C on yeast–mannitol broth (YMB) agar plates. Growth was assessed in parallel with other closely related type strains, *M.*

Table 2. Phenotypic characteristics of strain 10.2.2^T in comparison to closely related *Mesorhizobium* species

Strains: 1, 10.2.2^T; 2, 10.2.3; 3, *Mesorhizobium loti* NZP 2213^T [8, 42, 43]; 4, *Mesorhizobium cantuariense* ICMP 19515^T [44, 45]; 5, *Mesorhizobium ciceri* UMP-Ca7^T [8, 43, 45]. –, No growth observed; +, growth observed; NT, not tested; +^{W}, positive weak growth observed. Results denoted with * indicate that the information was acquired through the literature.

Characteristic	1	2	3	4	5
Assimilation of:					
Citrate	–	–	–	–	–
L-Lysine	+	+	+	+	+
D-Mannose	+	+	+	+	+
D-Serine	–	–	–	–	–
D-Mannitol	+	+	+	+	+
L-Alanine	+	+	+	+	–
Temperature for growth (°C):					
4	–	–	–	–	–
15	W	W	W	W	W
28	+	+	+	+	+
37	–	–	–	–	–
Incubation time (days)	3–5	3–5	3–5	7–9	3–5
pH growth range (at 28 °C)	above 4–10	above 4–10	4–8	above 4–10	4–10
Max. NaCl for growth (%)	1	1	1	1	1.5
Antibiotic resistance:					
Chloramphenicol	–	–	–	+	+
Phosphomycin	+	+	+	+	+
Spectinomycin	+^{W}	+^{W}	+	–	+
Ampicillin	–	–	+	–	–
Kanamycin	+	+	+	–	+
Gentamicin	–	–	–*	–*	–*
Streptomycin	–	NT	+*	–*	NT
Carbenicillin	–	NT	NT	NT	+*
Neomycin	–	NT	NT	NT	NT
Tetracycline	–	NT	NT	NT	NT
Rifampicin	–	NT	NT	NT	NT

loti NZP 2213^T, *M. ciceri* UMP Ca7^T, and *M. cantuariense* ICMP 19515^T, the latter of which only grew moderately sized colonies after 7–9 days. Tolerance to different stresses was tested by growing bacteria for 3 days in liquid YMB and diluting them to an OD_{600} of 0.1. 20 µl of culture was dropped onto YMB agar plates in duplicate in two independent experiments. Salinity tolerance was determined by using YMB agar supplemented with different concentrations of NaCl (0.01, 0.1, 0.5, 1.0, 1.5, 2.0, 3.0 and 5.0% NaCl). Isolates 10.2.2^T and 10.2.3 grew up to concentrations of 1% NaCl in contrast to *M. ciceri* UMP Ca7^T, which was able to grow up to 1.5% NaCl.

In addition, both isolates grew on YMB plates with pH that ranged from above 4 to 10 in contrast to *M. loti* NZP 2213^T, which grew in a pH range of 4–8 (Table 2).

The antibiotic resistance of each strain was investigated by growing them on YMB plates supplemented with either 34 µg ml^{-1} chloramphenicol, 15 µg ml^{-1} phosphomycin, 100 µg ml^{-1} ampicillin, 100 µg ml^{-1} spectinomycin or 50 µg ml^{-1} kanamycin. Isolates 10.2.2^T and 10.2.3 were sensitive to chloramphenicol while *M. canturiense* ICMP 19515^T and *M. ciceri* UMP Ca7^T were not. They were also

sensitive to ampicillin unlike *M. loti* NZP 2213^T, which was not. They also showed weak resistance to spectinomycin, which varied from the strong growth seen in *M. loti* NZP 2213^T and *M.ciceri* UMP Ca7^T, and no growth seen in *M. cantauriense* ICMP 19515^T. Strain 10.2.2^T also showed sensitivity to 100 µg ml^{-1} streptomycin, 50 µg ml^{-1} carbenicillin, 50 µg ml^{-1} neomycin, 5 µg ml^{-1} tetracycline, 25 µg ml^{-1} gentamicin and 50 µg ml^{-1} rifampicin.

Growth at particular temperatures was observed by inoculating each strain on YMB plates and incubating at either 4, 15, 28 or 37 °C. All strains grew best at 28 °C, weakly at 15 °C and not at all at 4 and 37 °C (Table 2).

An investigation of the utilization of several substrates as sole carbon sources was conducted on each strain on YM agar that had D-mannitol substituted with equal amounts of either citrate, D-serine, L-lysine, L-alanine or D-mannose (Table 2). All strains grew on L-lysine and D-mannose, while all but *M. cantuariense* ICMP 19515 were able to utilize L-alanine. Strain 10.2.2^T's carbon utilization was further tested with the API 20 NE kit following the manufacturer's instructions. Briefly, bacteria cultures were grown for 2 days in liquid YMB and diluted to an OD_{600}=0.1. After 96 h, growth was assessed visually. Strain 10.2.2 exhibited strong growth using D-glucose, *N*-acetyl-glucosamine or maltose as a carbon source and weaker growth when utilizing L-arabinose (Table 2).

The analysis of whole-cell fatty acids was conducted by the DSMZ as described previously [39–41]. Bacteria were grown in liquid YMB for 2 days at 28 °C. Biomass was collected and freeze-dried. The fatty acid composition was similar to other *Mesorhizobium* species and included 13.14% 11-methyl $C_{18:1}\omega7c$, 0.92% $C_{15:0}$ anteiso, 16.95% $C_{16:0}$, 3.59% $C_{17:0}$ iso, 42% $C_{18:1}\omega7c$, 7.31% $C_{18:0}$ and 16.09% $C_{19:0}$ cyclo ω8*c*.

Based on the results of phylogenetic analysis, phenotypic characteristics, and chemotaxonomic results, strain 10.2.2^T represents a novel species of the genus *Mesorhizobium*, for which the name *Mesorhizobium norvegicum* sp. nov. is proposed.

DESCRIPTION OF *MESORHIZOBIUM NORVEGICUM* SP. NOV.

Mesorhizobium norvegicum (nor.ve′gi.cum. N.L. neut. adj. *norvegicum* pertaining to Norway).

Colonies grown on YMB agar are circular, convex and slimy. Growth occurs at 28 °C in 0–1% NaCl and in a pH range from above 4 to 10. Cells are resistant to kanamycin and phosphomycin, weakly resistant to spectinomycin, and sensitive to streptomycin, carbenicillin, neomycin, tetracyclin, chloramphenicol, ampicillin, gentamicin and rifampicin. Cells assimilate D-glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetyl-glucosamine, maltose, L-lysine and L-alanine. Major fatty acids are 11-methyl $C_{18:1}\omega7c$, $C_{16:0}$, $C_{18:1}\omega7c$, $C_{18:0}$ and $C_{19:0}$ cyclo ω8*c*. The type strain, 10.2.2^T (=DSM 108834^T=LMG 31153^T), was isolated from a nodule of a *Lotus corniculatus* plant growing in Norway. The genome of the type strain is 8.27 Mbp and has a G+C content of 62.4 mol%.

Funding information
This work was funded by the German Research Foundation (Grant MA7269–1). T. K. was supported by the German Academic Exchange Service Study Scholarship for Graduates of All Disciplines 2016–2018 (Grant 57214223).

Acknowledgements
We thank Martin Parniske for providing isolates 10.2.2^{T}and 10.2.3, the Genetics Sequencing Service for sequencing of the genomes, and the DSMZ for conducting the fatty acid analyses.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References

1. Fowler D, Coyle M, Skiba U, Sutton MA, Cape JN *et al*. The global nitrogen cycle in the twenty-first century. *Philos Trans R Soc Lond B Biol Sci* 2013;368:20130164.
2. Roberts B. Biological nitrogen fixation. *Annu Rev Plant Physiol* 1993;29:263–276.
3. Bohlool BB, Ladha JK, Garrity DP, George T. Biological nitrogen fixation for sustainable agriculture: a perspective. *Plant Soil* 1992;141:1–11.
4. Considine MJ, Siddique KHM, Foyer CH. Nature's pulse power: legumes, food security and climate change. *J Exp Bot* 2017;68:1815–1818.
5. Stagnari F, Maggio A, Galieni A, Pisante M. Multiple benefits of legumes for agriculture sustainability: an overview. *Chem Biol Technol Agric* 2017;4:2.
6. United Nations. Transforming our world: the 2030 agenda for sustainable development. *UN DESA* 2015.
7. Brenner DJ, Krieg NR, Staley JT. Bergey's manual of systematic bacteriology. *Volume 2 : The Proteobacteria. Bergeys Manual of Systematic Bacteriology Volume 1*, 100; 2005.
8. Jarvis BDW, Van Berkum P, Chen WX, Nour SM, Fernandez MP *et al*. Transfer of *Rhizobium loti, Rhizobium huakuii, Rhizobium ciceri, Rhizobium mediterraneum*, and *Rhizobium tianshanense* to *Mesorhizobium* gen. nov. *Int J Syst Bacteriol* 1997;47:895–898.
9. Laranjo M, Alexandre A, Oliveira S. Legume growth-promoting rhizobia: an overview on the *Mesorhizobium* genus. *Microbiol Res* 2014;169:2–17.
10. Young PJW, Haukka KE. Diversity and phylogeny of rhizobia. *New Phytologist* 1996;133:87–94.
11. Andrews M, Andrews ME. Specificity in Legume-Rhizobia symbioses. *Int J Mol Sci* 2017;18:E705.
12. Maxted N, Ambrose M. Plant genetic resources of legumes in the Mediterranean. In: Maxted N and Bennett SJ (editors). *Plant Genetic Resources of Legumes in the Mediterranean*. Dordrecht: Springer Netherlands; 2001. pp. 181–190.
13. Jarvis BDW, Pankhurst CE, Patel JJ, Ioti R. A new species of legume root nodule bacteria. *Int J Syst Evol* 1982;32:378–380.
14. Marcos-García M, Menéndez E, Ramírez-Bahena MH, Mateos PF, Peix Álvaro *et al*. *Mesorhizobium helmanticense* sp. nov., isolated from *Lotus corniculatus* nodules. *Int J Syst Evol Microbiol* 2017;67:2301–2305.
15. Lorite MJ, Flores-Félix JD, Peix Álvaro, Sanjuán J, Velázquez E. *Mesorhizobium olivaresii* sp. nov. isolated from *Lotus corniculatus* nodules. *Syst Appl Microbiol* 2016;39:557–561.
16. Gossmann JA, Markmann K, Brachmann A, Rose LE, Parniske M. Polymorphic infection and organogenesis patterns induced by a *Rhizobium leguminosarum* isolate from *Lotus* root nodules are determined by the host genotype. *New Phytol* 2012;196:561–573.

17. **Röhm M**, **Werner D**. Nitrate levels affect the development of the black locust-*Rhizobium* symbiosis. *Trees* 1991;5:227–231.
18. **Liang J**, **Hoffrichter A**, **Brachmann A**, **Marín M**. Complete genome of *Rhizobium leguminosarum* Norway, an ineffective *Lotus* microsymbiont. *Stand Genomic Sci* 2018;13:36.
19. **William S**. **Feil HAC**. 2012. Bacterial genomic DNA isolation using CTAB. Joint genome Institute, Walnut Creek, CA. http://jgi.doe.gov/wp-content/uploads/2014/02/JGI-Bacterial-DNA-isolation-CTAB-Protocol-2012.pdf
20. **Lee I**, **Chalita M**, **Ha SM**, **Na SI**, **Yoon SH** ***et al***. ContEst16S: an algorithm that identifies contaminated prokaryotic genomes using 16S RNA gene sequences. *Int J Syst Evol Microbiol* 2017;67:2053–2057.
21. **Gurevich A**, **Saveliev V**, **Vyahhi N**, **Tesler G**. QUAST: quality assessment tool for genome assemblies. *Bioinformatics* 2013;29:1072–1075.
22. **Médigue C**, **Calteau A**, **Cruveiller S**, **Gachet M**, **Gautreau G** ***et al***. MicroScope—an integrated resource for community expertise of gene functions and comparative analysis of microbial genomic and metabolic data. *Brief Bioinform* 2017;44.
23. **Vallenet D**, **Belda E**, **Calteau A**, **Cruveiller S**, **Engelen S** ***et al***. MicroScope-an integrated microbial resource for the curation and comparative analysis of genomic and metabolic data. *Nucleic Acids Res* 2013;41:D636–D647.
24. **Vallenet D**, **Engelen S**, **Mornico D**, **Cruveiller S**, **Fleury L** ***et al***. Microscope: a platform for microbial genome annotation and comparative genomics. *Database 2009* 2009:bap021.
25. **Klioutchnikov G**, **Kriventseva EV**, **Zdobnov EM**, **Seppey M**, **Waterhouse RM** ***et al***. BUSCO applications from quality assessments to gene prediction and phylogenomics. *Mol Biol Evol* 2017;35:543–548.
26. **Simão FA**, **Waterhouse RM**, **Ioannidis P**, **Kriventseva EV**, **Zdobnov EM**. BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics* 2015;31:3210–3212.
27. **Kaneko T**, **Nakamura Y**, **Sato S**, **Asamizu E**, **Kato T** ***et al***. Complete genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*. *DNA Res* 2000;7:331–338.
28. **Marcos-García M**, **Menéndez E**, **Cruz-González X**, **Velázquez E**, **Mateos PF** ***et al***. The high diversity of *Lotus corniculatus* endosymbionts in soils of northwest Spain. *Symbiosis* 2015;67:11–20.
29. **Katoh K**, **Rozewicki J**, **Yamada KD**. MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Brief Bioinform* 2017;30.
30. **Stamatakis A**. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 2014;30:1312–1313.
31. **Miller MA**, **Pfeiffer W**, **Schwartz T**. *The CIPRES Science Gateway*. New York, USA: ACM Press; 2012.
32. **Lee I**, **Ouk Kim Y**, **Park SC**, **Chun J**. OrthoANI: an improved algorithm and software for calculating average nucleotide identity. *Int J Syst Evol Microbiol* 2016;66:1100–1103.
33. **Konstantinidis KT**, **Tiedje JM**. Genomic insights that advance the species definition for prokaryotes. *Proc Natl Acad Sci USA* 2005;102:2567–2572.
34. **Sullivan JT**, **Trzebiatowski JR**, **Cruickshank RW**, **Gouzy J**, **Brown SD** ***et al***. Comparative sequence analysis of the symbiosis island of *Mesorhizobium loti* strain R7A. *J Bacteriol* 2002;184:3086–3095.
35. **Rogel MA**, **Ormeño-Orrillo E**, **Martinez Romero E**. Symbiovars in rhizobia reflect bacterial adaptation to legumes. *Syst Appl Microbiol* 2011;34:96–104.
36. **Lorite MJ**, **Estrella MJ**, **Escaray F**, **Sannazzaro A**, **Videira e Castro IM**. The Rhizobia-Lotus symbioses: deeply specific and widely diverse 2055;2018:9.
37. **Kumar S**, **Stecher G**, **Li M**, **Knyaz C**, **Tamura K**. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol* 2018;35:1547–1549.
38. **Stothard P**. The sequence manipulation suite: JavaScript programs for analyzing and formatting protein and DNA sequences. *Biotechniques* 2000;28:1102–1104.
39. **Kämpfer P**, **Kroppenstedt RM**. Numerical analysis of fatty acid patterns of coryneform bacteria and related taxa. *Can J Microbiol* 1996;42:989–1005.
40. **Kuykendall LD**, **Roy MA**, **O'NEILL JJ**, **Devine TE**, **Acids F**. Fatty acids, antibiotic resistance, and deoxyribonucleic acid homology groups of *Bradyrhizobium japonicum*. *Int J Syst Bacteriol* 1988;38:358–361.
41. **Miller L T**. A single derivatization method for bacterial fatty acid methyl esters including hydroxy acids. *Journal of Clinical Microbiology* 1982;16:584–586.
42. **Martínez-Hidalgo P**, **Ramírez-Bahena MH**, **Flores-Félix JD**, **Rivas R**, **Igual JM** ***et al***. Revision of the taxonomic status of type strains of *Mesorhizobium loti* and reclassification of strain USDA 3471T as the type strain of *Mesorhizobiumerdmanii* sp. nov. and ATCC 33669T as the type strain of *Mesorhizobiumjarvisii* sp. nov. *Int J Syst Evol Microbiol* 2015;65:1703–1708.
43. **Nour SM**, **Fernandez MP**, **Normand P**, **Cleyet-Marel JC**. *Rhizobium ciceri* sp. nov., consisting of strains that nodulate chickpeas (*Cicer arietinum* L.). *Int J Syst Bacteriol* 1994;44:511–522.
44. **De Meyer SE**, **Tan HW**, **Andrews M**, **Heenan PB**, **Willems A**. *Mesorhizobium calcicola* sp. nov., *Mesorhizobium waitakense* sp. nov., *Mesorhizobium sophorae* sp. nov., *Mesorhizobium newzealandense* sp. nov. and *Mesorhizobium kowhaii* sp. nov. isolated from Sophora root nodules. *Int J Syst Evol Microbiol* 2016;66:786–795.
45. **De Meyer SE**, **Willems A**, **Wee Tan H**, **Andrews M**, **Heenan PB**. *Mesorhizobium waimense* sp. nov. isolated from *Sophora longicarinata* root nodules and *Mesorhizobium cantuariense* sp. nov. isolated from *Sophora microphylla* root nodules. *Int J Syst Evol Microbiol* 2015;65:3419–3426.

Publication II:

Microbiome profiling reveals that *Pseudomonas* antagonises parasitic nodule colonisation of cheater rhizobia in *Lotus*

Research

New Phytologist

Microbiome profiling reveals that *Pseudomonas* antagonises parasitic nodule colonisation of cheater rhizobia in *Lotus*

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Received: *22 October 2021*
Accepted: *11 January 2022*

New Phytologist (2022) **234:** 242–255
doi: 10.1111/nph.17988

Key words: ineffective nodules, *Lotus*, *Mesorhizobium*, microbiome, *Pseudomonas*, root–nodule symbiosis.

Summary

- Nodule microbiota are dominated by symbiotic nitrogen-fixing rhizobia, however, other non-rhizobial bacteria also colonise this niche. Although many of these bacteria harbour plant-growth-promoting functions, it is not clear whether these less abundant nodule colonisers impact root–nodule symbiosis.
- We assessed the relationship between the nodule microbiome and nodulation as influenced by the soil microbiome, by using a metabarcoding approach to characterise the communities inside nodules of healthy and starved *Lotus* species. A machine learning algorithm and network analyses were used to identify nodule bacteria of interest, which were re-inoculated onto plants in controlled conditions to observe their potential functionality.
- The nodule microbiome of all tested species differed according to inoculum, but only that of *Lotus burttii* varied with plant health. Amplicon sequence variants representative of *Pseudomonas* species were the most indicative non-rhizobial signatures inside healthy *L. burttii* nodules and negatively correlated with *Rhizobium* sequences. A representative *Pseudomonas* isolate co-colonised nodules infected with a beneficial *Mesorhizobium*, but not with an ineffective *Rhizobium* isolate and another even reduced the number of ineffective nodules induced on *Lotus japonicus*.
- Our results show that nodule endophytes influence the overall outcome of the root–nodule symbiosis, albeit in a plant host-specific manner.

Introduction

Leguminous plants have evolved a mutualistic interaction with nitrogen-fixing rhizobia in which the bacteria are hosted and nourished in root organs called nodules in exchange for ammonia. This so-called root–nodule symbiosis is initiated by a two-way signalling between the symbiosis partners, which activates distal cell divisions in the root cortex and culminates in the formation and infection of nodules (Venado *et al.*, 2020). Here the bacteria differentiate into plant-dependent, nitrogen-fixing endosymbiotic bacteroids (Kereszt *et al.*, 2011). The fixation of nitrogen is an energetically expensive process for the host that requires at least 16 ATP molecules per N_2 molecule to fuel the nitrogenase enzyme produced by the rhizobia (Seefeldt *et al.*, 2009). Therefore, to prevent infection of the carbon-rich nodules by pathogens, host plants have evolved complex recognition mechanisms that ensure symbiotic specificity (Wang *et al.*, 2012).

Root–nodule symbiosis is highly species specific and many plants will only form an effective symbiosis with a narrow range of rhizobia (Remigi *et al.*, 2016). Even within these pairings there is variation in nitrogen fixation efficiency (Schumpp & Deakin, 2010). Some bacteria can also nodulate plants and not fix any nitrogen at all (Sachs & Simms, 2008). Examples of ineffective nitrogen fixation have been described after the introduction of crop legumes into areas where native legumes previously grew. For instance, inefficient nitrogen fixation occurs in fields where perennial and annual clovers co-exist (Howieson *et al.*, 2005). Native rhizobial species associated with native legumes can outcompete inoculant strains (Streeter, 1994). In extreme cases, endogenous rhizobia can completely block the nodulation of introduced rhizobia. For example, the nodulation of the pea cultivars Afghanistan and Iran by rhizobial inoculants is suppressed in natural soils by the presence of a non-nodulating strain (Winarno & Lie, 1979). This suggests that interactions of the soil microbiota with the host plant are critical for the establishment of efficient nodules. However, we are far from understanding what factors determine the success of single microbes that compete for resources at the plant soil interface, in particular nodule endophytes and how these affect the outcome of the symbiosis.

There is clear evidence to suggest that the host controls the makeup of the microbiota in its vicinity. *Lotus japonicus* selects for

a broad taxonomic range of bacteria, in addition to the symbiont, within the rhizosphere, endosphere and the nodule. This selectivity filters the diverse soil microbiome into a distinct and taxonomically narrow community within the nodule (Zgadzaj *et al*., 2016). Despite this selective pressure, nonnodulating bacteria, such as *Pseudomonas* sp., *Klebsiella* sp. and *Rhodococcus* sp. have been isolated from plant nodules (Ibáñez *et al*., 2009; Ampomah & Huss-Danell, 2011; Martínez-Hidalgo & Hirsch, 2017). Although these isolates do not directly nodulate the plant, they contribute to plant growth in some ways, such as increasing the availability of soluble phosphate and producing plant compounds beneficial for plant growth such as siderophores and indoleacetic acid (Dey *et al*., 2004; Malik & Sindhu, 2011; Zhao *et al*., 2013). In addition, non-Rhizobiales microbes found in nodules of *Medicago truncatula* produce antimicrobial compounds that may shape the community and the overall function of the nodule microbiome (Hansen *et al*., 2020). Microbe–microbe interactions could also impart an effect on the overall functionality of the symbiosis, for instance via antimicrobial activity (Tyc *et al*., 2014), suppression of plant pathogens (Gu *et al*., 2020) or by horizontal gene transfer (Cytryn, 2013). Although these complex interactions could dictate the effectiveness and specificity of the symbiosis, little information is known about how rhizobia interact with other members of the nodule microbiota.

In this work, we determined the nodule microbiome of three *Lotus* species upon inoculation with soil suspensions that led to the growth of either starved or healthy plants. We used metabarcoding-based high-throughput sequencing to characterise the microbiome in nodule samples that varied in plant species origin, soil inocula and plant health. Network analyses and machine learning algorithms identified microbiome members specifically associated to nodules of healthy, but not of starved *Lotus burttii* plants. Tripartite interactions between rhizobia, nodule endophytes and the host were further investigated in co-inoculation assays. Our results show that although root–nodule symbiosis is a binary interaction, there are other nodule microbes that modulate this mutualism.

Materials and Methods

Soil collection and inoculum preparation

Soil samples were collected from two neighbouring sites in a semiurban area south west of Munich, Germany. Site 1 (48°06′29.9″N, 11°27′38.9″E) has consistently been home to wild *Lotus corniculatus*, whereas site 2 (48°06′33.2″N, 11°27′41.4″ has been subjected to tilling and physical disturbance and did not contain *Lotus* plants at the time of collection. Soil samples were taken from the top layer (0–20 cm deep) after plant material was removed from the site in May 2019 and October 2018. Physicochemical property measurements of each soil were performed by AGROLAB Agrarzentrum GmbH (Landshut, Germany). Soil samples were sieved to remove stones and plant material with a 2 mm sieve, mixed 1 : 5 with a nitrogen-limiting Fabaceae (FAB) liquid medium, and stirred for 2 h. Soil particulate matter was removed by centrifugation at 1000 ***g*** for 5 min. Soil suspensions were used as inputs and a quantitative PCR (qPCR) was run to compare the quantity of soil bacteria present in both soil suspensions inputs.

Plant growth and inoculation conditions

Lotus burttii B-303 (seed bag no. 91105), *L. japonicus* Gifu B-129 (seed bag no. 110913) and *L. corniculatus* cv Leo (Andreae Saaten, Regensburg, Germany) seeds were scarified and then sterilised by incubation in a sterilising solution (1.2% NaOCl, 1% SDS) for 8 min before being washed three times with sterile water. Seeds were then soaked in sterile water for 2–3 h and germinated on 0.5 B5 agar medium (Gamborg *et al*., 1968) for 3 d in dark followed by 3 d in a long-day photoperiod (16 h : 8 h, light : dark) at 24°C. Seedlings were then transferred into sterilised tulip-shaped Weck jars (10 seedlings per jar) containing 300 ml of a sand : vermiculite mix (1 : 2) and supplemented with 40 ml of a low nitrogen FAB medium, to create nitrogen-limiting growth conditions as mentioned above (Liang *et al*., 2019). Jars were sealed with micropore tape to create a closed system. Seedlings were left to recover for 2 d in a long-day photoperiod. After the 2-d recovery, each seedling was inoculated with 1 ml of soil suspension. *Lotus burttii* and *L. japonicus* treatments consisted of 150 plants from three independent experiments, and *L. corniculatus* treatments consisted of 50 plants per condition from one independent experiment.

Harvesting, phenotyping and nodule surface sterilisation

Plants were harvested and phenotyped 5 wk post inoculation across five independent experiments. Shoot length, shoot dry weight, nodule number, nodule colour and plant health were recorded. Nodules were classified as pink or white, which indicated the presence or absence of leghaemoglobin, respectively, a prerequisite for, but not a guarantee of, nitrogen fixation (Downie, 2005). Roots were removed from shoots and sonicated using the Bioruptor® (Diagenode, Seraing, Belgium) twice for 15 min. Nodules from three or four plants were excised and pooled based on similarity of plant shoot and nodule phenotype. Pink and white nodules were collected separately. Pooled nodules were treated with 70% ethanol for 1 min followed by 2% NaOCl for 2.5 min. Nodules were then washed with sterile water eight times and after the removal of the final water wash, samples were snap frozen in liquid nitrogen. The final wash was plated onto 20Q agar supplemented with 3.8% w/v mannitol (modified from Werner *et al*., 1975) to assess sterilisation.

DNA extraction

Nodule samples were homogenised six times in a Mixer Mill 400 (Retsch, Haan, Germany) at a frequency of 30 s^{-1} for 1 min. DNA was then extracted according to a modified protocol from Töwe *et al*. (2011). For extraction of DNA from the inputs, soil suspensions were centrifuged at 5000 ***g*** and DNA from pellets was extracted according to the CTAB method described by the Doe Joint Genomics Institute (William *et al*., 2012). The

concentrations of extracted DNA samples were quantified using a Qubit 2.0® fluorometer (Invitrogen, Carlsbad, CA, USA).

Quantitative PCR

Quantitative PCR was performed using the forward primer (FP) *16S rDNA* (5′-GGTAGTCYAYGCMSTAAACG-3′) and reverse primer (RP) *16S rDNA* (5′-GACARCCATGCASCACCTG-3′) primers (Bach *et al.*, 2002). The 25-µl PCR mixture contained 12.5 µl SYBR Green, 2 µl template DNA, 7.5 µl Milli-Q water, 1 µl 10 µM of primers and 1 µl 15% bovine serum albumin (BSA). The mixture was amplified using a CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA) under the following conditions: template was denatured at 94°C for 10 min before 40 cycles of 95°C for 20 s, 57°C for 30 s and 72°C for 45 s, followed by dissociation curve steps of 95°C for 15 s, 60°C for 30 s and 95°C for 15 s. Quantification of the *16S* rRNA gene molecules was correlated with a calibration curve constructed with known amounts of a *16S* rRNA gene standard plasmid constructed of a *Mesorhizobium septentrionale 16S* rRNA gene sequence cloned into a pUC57 plasmid.

Amplification, library preparation and sequencing

To determine bacterial diversity, a metabarcoding approach was utilised. The hypervariable region V3–V4 of the *16S* rRNA gene was amplified using universal bacterial primers 335F (5′-CADACTCCTACGGGAGGC-3′) and 769R (5′-ATCCTGTTTGMTMCCCVCRC-3′) fused to Illumina adapters. The primers were specific for bacterial DNA and did not amplify plastidial and mitochondrial plant DNA (Dorn-In *et al.*, 2015). Amplification reaction volumes were 25 µl using 1 unit of Phusion polymerase, 5 µl 5× High-Fidelity Phusion buffer, 7.5 µl of 1% BSA, 0.5 µl of 10 mM dNTPs, 0.5 µl of 50 mM $MgCl_2$, 0.5 µl of 10 pmol μl^{-1} primer and 5 ng of template DNA. The assay was conducted in triplicate under the following conditions: template was denatured at 98°C for 1 min, then 25 cycles of 98°C for 10 s, 55°C for 30 s and 72°C for 30 s, followed by a final step at 72°C for 5 min. PCR products were verified via gel electrophoresis, pooled, and cleaned using CleanPCR beads (CleanNA, Waddinxveen, the Netherlands). Fragments were then indexed with 10 nucleotide barcode sequences using the Nextera XT Index Kit v.2 Set D primers (Illumina, San Diego, CA, USA). Indexing PCR reactions were run in triplicate with a volume of 25 µl using 12.5 µl NEB Next High-Fidelity Master Mix, 2.5 µl of each delegated primer and 20 ng of amplicon under the following conditions: template was denatured at 98°C for 30 s, then eight cycles of 98°C for 10 s, 55°C for 30 s and 72°C for 30 s, followed by a final step at 72°C for 5 min. PCR products were pooled and cleaned with CleanPCR beads (CleanNA). Quantification and quality control were conducted using an AATI Fragment Analyser (Santa Clara, CA, USA). All samples were pooled at an equimolar concentration for paired-end 2 × 300-bp sequencing via the MiSeq system (Illumina) using the MiSeq Reagent Kit v.3 (600 cycles), as per the manufacturer's recommendation.

Sequence and statistical analysis

An average of *c.* 141 000 raw Illumina reads per sample were obtained, which were then demultiplexed and had adapter and barcode sequences removed using CUTADAPT v.3.1 (Martin, 2011). Reads were then trimmed, merged and filtered using DADA2 (Callahan *et al.*, 2016) in R. The criteria for filtering were minimum lengths of 280 bp for the forward reads and 160 bp for the reverse, as these lengths corresponded to a minimum quality score of 25. Merged sequences had chimeras and chloroplastic and mitochondrial sequences removed. Amplicon sequence variants (ASVs) were assigned in R using the Silva database v.132 (Quast *et al.*, 2012).

The PHYLOSEQ v.1.26.1 package in the R (McMurdie & Holmes, 2013) pipeline was used to infer alpha diversity of ASVs rarefied corresponding to the sample with the lowest number of reads. Multidimensional Scaling using Bray–Curtis (Bray & Curtis, 1957) distance was performed using the PHYLOSEQ v.1.26.1 package in R (McMurdie & Holmes, 2013) to assess the beta diversity of microbial communities. Comparisons were visualised using GGPLOT2 (Wickham, 2009) in R and tested for statistical significance (*adonis* test, $P < 0.01$) via permutational multivariate analysis of variance (PERMANOVA) utilising 999 permutations in the VEGAN package (Oksanen *et al.*, 2018). Relative abundance of each genera per sample was calculated using transformed count data. To further specify the composition of the sample microbiome the relative abundance of the most prevalent ASVs (abundance > 0.1%) was calculated for each sample. All abundance levels were calculated using the PHYLOSEQ v.1.26.1 package (McMurdie & Holmes, 2013) in R.

Machine learning model

A support vector machine learning model by svm.SVC (kernel=linear) in PYTHON SCIKIT-LEARN (Pedregosa *et al.*, 2011) was used to discriminate between starved and healthy plant samples of *L. burttii* on relative abundance filtered ASVs using five-fold cross-validation. The ASV tables were filtered to ASVs present at ≥ 50 reads in soil suspension 2 inoculated *L. burttii* nodule samples. The *svm.SVC.coef* function was used to calculate the coefficient value of the ASVs. These values were then used to identify signature ASVs characteristic of certain sample types. The model was trained with 70% of the data and evaluated by 30% of the data five times (mean of accuracy = 0.89) with the average coefficient value of each ASV being used to select for important features.

Microbial correlation networks

Filtered ASV tables comprised of samples of *L. burttii* inoculated with soil suspension 2 (ASV raw abundances) were used to calculate microbial correlation networks among ASVs using the SPARCC (Friedman & Alm, 2012) algorithm in FASTSPAR (Watts *et al.*, 2019). This algorithm uses log-ratio variances of ASV fractions to calculate pairwise correlations between ASVs in an iterative manner. The filtered tables were used to calculate the

correlation between ASVs using the FastSpar implementation and the default parameters. Pseudo *P*-values were inferred from 1000 bootstraps. Only correlations with $P < 0.01$ were kept for further analyses. Network visualisation was performed in Cytoscope v.3.8.2 (Shannon *et al.*, 2003). Analysis of interactions between and within ASVs of different genera were carried out using the same methods.

Isolation of strains

Strains were isolated from crushed nodules on a variety of media. Nodules from either *L. burttii*, *L. corniculatus* or *L. japonicus* inoculated with either soil suspension 1 or 2 were sterilised as described above (see 'Harvesting and nodule sterilisation' in the Materials and Methods section). Individual nodules were crushed in 10 mM $MgSO_4$ and the content was then spread onto 20Q agar plates supplemented with mannitol, lysogeny broth (LB) (Bertani, 1951), yeast mannitol (YM) (Vincent, 1970), *Pseudomonas* minimal medium (PMM) (Sandman & Ecker, 2014), and tryptone soy (TS) (Sigma, Darmstadt, Germany). Plates were incubated at 28°C for up to 3 wk and further isolation of single colonies was carried out 7–9 times until pure cultures were attained. The taxonomy of each strain was determined by amplifying the *16S* rRNA gene using primers 41f (5′-GCTCAGATTGAACGCTGGCG-3′) and 1488r (5′-CGGTTACCTTGTTACGACTTCACC-3′) (Herrera-Cervera *et al.*, 1999) and Phusion polymerase. Amplicons were purified using a 1 : 0.8 ratio of PCR product to CleanPCR beads (CleanNA) and sequenced using *16S* rRNA gene-specific primers, 41f and 1488r (Herrera-Cervera *et al.*, 1999), by Sanger sequencing. Sequences were aligned to DNA sequences from the NCBI Nucleotide collection online database using Blastn (Altschul *et al.*, 1990). The sequences of the isolates were aligned with ASV sequences using CLC Main Workbench 7 (Qiagen, Hilden, Germany). Strains were stored in 40% glycerol at −80°C.

Isolate inoculations

Bacteria were streaked and grown until single colonies formed. Single colonies were inoculated into 2 ml of the appropriate liquid medium and grown at 28°C for 2, 3 or 5 d for *Pseudomonas* sp. strains, *Rhizobium* sp. BW8-2 and *Mesorhizobium* sp. Qb1E3-1, respectively. Bacteria were then washed in sterile water and resuspended in FAB medium to allow for a final OD_{600} of 0.005. Each plant was inoculated with 1 ml of bacterial suspension. Plants were prepared as described above.

Conjugation

Strains used in this work are listed in Supporting Information Table S1. *Escherichia coli* ST18 transformed with pFAJ-GFP and pFAJ-*Ds*Red plasmids (Kelly *et al.*, 2013) and *E. coli* S17.1 transformed with pABC-Cerulean were grown at 37°C overnight in LB supplemented with appropriate antibiotics. *Pseudomonas* sp. Lb2C2, *Rhizobium* sp. BW8-2 and *Mesorhizobium* sp. Qb1E3-1 were grown at 28°C in 20Q liquid with the appropriate antibiotics for 2, 3 and 5 d, respectively. Conjugations were conducted as in Liang *et al.* (2019). Successful conjugation was confirmed via fluorescence microscopy and *16S* rRNA gene sequencing.

Section preparation and microscopy

Lotus burttii seeds were sterilised, germinated, potted and inoculated with fluorescent strains as described above (see 'Plant growth and inoculation conditions' and 'Isolate inoculations' in the Materials and Methods section). Plants were harvested 2 wk after inoculation and the nodules were excised and embedded in 6% low melting agarose. The nodules were then sliced into 100 µm-thick sections using a VT1000S vibratome (Leica Biosystems, Wetzlar, Germany) and visualised with a TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with a ×20 HCX PL APO water immersion lens. GFP and Cerulean were excited with an argon laser line at 488 and 433 nm, and the emissions were detected at 492–515 and 455–474 nm, respectively. *Ds*Red was excited with a diode pumped solid-state laser at 561 nm and detected at 580–620 nm.

Statistical analyses

The nodule and root phenotype of plants inoculated with nodule isolates were recorded. Statistical significance was assessed using analysis of variance (ANOVA) and Tukey honestly significant difference (HSD) tests in R (Graves *et al.*, 2015).

Results

Species-specific effect of soil inoculum on *Lotus* plant growth

Two different soil suspensions were used to inoculate *L. burttii*, *L. japonicus*, and *L. corniculatus* plants. These *Lotus* species were selected as they all belonged to the *L. corniculatus* clade (Kramina *et al.*, 2016), but nodulated with a different range of microsymbionts (Gossmann *et al.*, 2012; Sandal *et al.*, 2012). The first soil (soil 1) was collected at a site that contained healthy wild growing *L. corniculatus* plants, while the second soil (soil 2) site contained no leguminous plants at all. The soils had minor differences in mineral content and grain size (Table S2). The quantity of soil bacteria present in the soil suspensions used as inputs was compared by qPCR. Soil suspension inputs 1 and 2 contained 1.62×10^5 and 2.28×10^5 molecules of the *16S* rRNA gene per nanogram of extracted DNA, respectively.

Lotus japonicus, *L. burttii* and, to a lesser extent, *L. corniculatus*, produced exclusively healthy plants (green leaves, elongated shoots) when inoculated with soil 1 suspension (Soil S1; Fig. 1). Contrastingly, there was marked variation in the shoot growth phenotype seen in all species when inoculated with the soil 2 suspension (Soil S2). Growing alongside the healthy plants was a large contingent of starved plants presenting with shorter shoots and yellow leaves (Fig. 1). Similar results were observed across five independent experiments (Fig. S1). Nodule number also varied dependent on soil suspension inoculum. Plants inoculated with soil 1 suspension

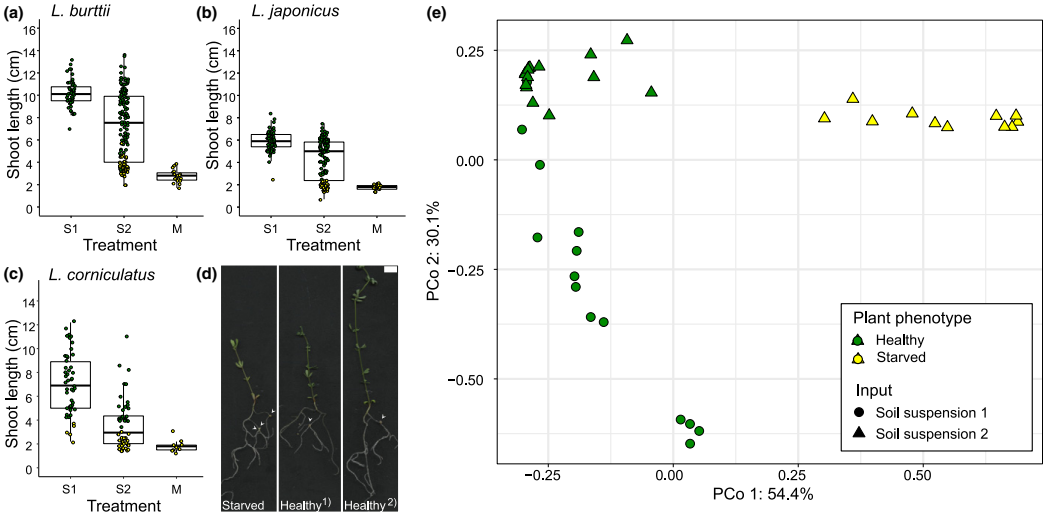

Fig. 1 Shoot growth phenotype of *Lotus* plants inoculated with Munich soil suspensions. Shoot growth quantification of *Lotus burttii* (a), *Lotus japonicus* (b) and *Lotus corniculatus* (c) plants 5 wk post inoculation with soil suspensions 1 (S1) and 2 (S2) and a mock (M) treatment. Green and yellow dots indicate plants with healthy and starved phenotypes, respectively. Box plots display the results of 50–150 plants per condition. The bold black line and the box depict the median and the interquartile range, respectively. In total, 49 mock treated plants were included. (d) Scanned images of *L. burttii* 5-wk post inoculation with soil suspension 2. Starved plants exhibited pale green leaves despite having nodules on their roots. The shoots of healthy dark green plants varied in length. Phenotypic variation is depicted in [1)] and [2)]. White arrowheads indicate the position of nodules on plant roots. Plots show the results from one representative experiment. Bar, 1 cm. (e) Principal coordinates analysis plot of *L. burttii* nodules based on beta diversity calculated using the Bray–Curtis dissimilarity index (Bray & Curtis, 1957) revealed a clustering of common sample types and a separation of dissimilar sample types.

consistently developed a higher number of nodules per plant across all species (Fig. S2a–c). Starved plants inoculated with the soil 2 suspension exhibited roots either with or without nodules. In *L. burttii*, 73.8% of starved plants contained nodules, while in *L. japonicus* and *L. corniculatus*, 45.2% and 59.3% exhibited nodules, respectively (Fig. S2d). However, the most striking difference was that in *L. burttii* 88.4% of the nodules on starved-nodulated plants were white, whereas in the other species most of the nodules were pink (Fig. S2e). These results showed that the microbiota of the soil 2 suspension is capable of mediating both effective and ineffective symbiosis, although the frequency at which each plant succumbs to an ineffective nodulation differs.

Richness, diversity and community structure of the *Lotus* nodule microbiome

The microbiome of an effective plant nodule is typically dominated by the respective symbiont, although there can also be colonisation by other microbes (Martínez-Hidalgo & Hirsch, 2017). To investigate how the nodule microbiota varied depending on the plant host, inoculum, and nodule phenotype we sequenced the microbiome of nodules collected from healthy and starved *Lotus* of different species inoculated with different soil suspensions. A variable region of the *16S* rRNA gene was sequenced and the output reads were processed, sorted into ASVs and assigned a taxonomy. ASVs were used as they provide a finer resolution than Operational Taxonomic Units (Callahan *et al.*, 2017), which is important as the 16S rRNA gene of some rhizobia, such as *Mesorhizobium*, can be more than 99% identical between different species (Marcos-García *et al.*, 2015). Sequencing produced 13 989 700 paired-end reads after quality filtering, which clustered into 67 442 unique ASVs. Sequence coverage varied between sample types with the nodule samples having an average of 148 679 reads per sample and the soil suspension input samples having an average of 67 618 reads per sample (Dataset S1). All rarefaction curves reached a saturation plateau (Fig. S3).

To assess the effect of the host genotype and the inoculum on the nodule microbiome diversity, the alpha and beta diversities of the different nodule samples from all three species were determined. Within sample variation (alpha diversity) was calculated using the Shannon diversity index, which was found to be much higher in the soil suspension input samples compared with the nodule samples (Fig. S4a). The soil suspensions 1 and 2 did not significantly vary in their alpha diversities (Welch two sample *t*-test, $P = 0.749$), although it was found that plants inoculated with soil 1 suspension produced nodules with a much higher alpha diversity compared with those inoculated with soil 2 suspension. This observation was most pronounced in *L. japonicus* and *L. corniculatus* (Fig. S4). A similar trend in alpha diversity was seen when considering observed ASVs (Fig. S4b).

To analyse the diversity between sample types (beta diversity), principal coordinate and PERMANOVA analyses were conducted using the Bray–Curtis dissimilarity. A global comparison of the nodule diversity showed an overall separation based on soil suspension input (Fig. S5; Soil S1 vs Soil S2, Pr(> *F*) = 0.001; Table S3), despite the two soil suspension inputs showing insignificant differences between one another (Soil S1 vs Soil S2 (input suspension), Pr(> *F*) = 0.072; Table S3). The most pronounced difference was between nodules of *L. burttii* plants. At the species level, *L. burttii* and *L. japonicus* showed a significant difference in beta diversity based on soil suspension input (*Lb* healthy plants – Soil S1 vs Soil S2, Pr(> *F*) = 0.001; *Lj* healthy plants – Soil S1 vs Soil S2, Pr(> *F*) = 0.002; Table S3). *Lotus burttii* nodules showed a significant difference in beta diversity based on plant health (*Lb* Soil S2 – healthy vs starved plants, Pr (> *F*) = 0.001; Fig. 1d; Table S3), however this was not the case in *L. japonicus* or *L. corniculatus* (*Lj* Soil S2 – healthy vs starved plants, Pr(> *F*) = 0.097; *Lc* Soil S2 – healthy vs starved plants, Pr (> *F*) = 0.742; Table S3). As a control, we compared the microbiome of laboratory grown *L. corniculatus* plants to the microbiome of nodules collected from *L. corniculatus* plants growing on site 1 (*Lc* Soil S1 – laboratory grown vs wild plants, Pr (> *F*) = 0.342) (Table S3). These did not significantly differ, supporting that nodules produced in this growth/inoculation system are representative of nodules grown in the wild.

Bacterial composition of the nodule microbiome

Both soil suspension types were dominated by Alphaproteobacteria and Gammaproteobacteria. To determine the bacterial composition of the nodule microbiome we estimated the relative abundance at an ASV level. The nodule microbiome of all *Lotus* species was dominated by ASVs belonging to the order Rhizobiales. Nodules of healthy plants were largely dominated by *Mesorhizobium*, independent of the host and soil suspension input. However, while nodules from plants inoculated with soil 1 suspension were colonised with a variety of different *Mesorhizobium* ASVs, the nodules of healthy plants inoculated with soil 2 suspension were almost exclusively colonised by *Mesorhizobium* ASV1 (Fig. 2). This disparity in *Mesorhizobium* ASV presence is despite the observation that there is no significant difference between the *Mesorhizobium* ASVs present in the two suspensions (*Meso.* Soil S1 vs *Meso.* Soil S2, *P*(> *F*) = 0.479). Nodules of starved *L. burttii* plants were largely colonised by bacteria belonging to what was taxonomically defined as *Allorhizobium–Neorhizobium–Pararhizobium–Rhizobium* and will be referred to as *Rhizobium* (Fig. 2a). This suggests that *L. burttii* plants are less selective compared with *L. corniculatus* and *L. japonicus* and develop an ineffective symbiosis with *Rhizobium* strains.

Pseudomonas are more prevalent in healthy plant nodules and negatively correlate with ineffective *Rhizobium*

Support Vector Machine (SVM) is a machine learning method used to separate a data set using a linear or nonlinear surface (Noble, 2006). In this instance we used a linear-kernel to transform the data and then based on this transformation defined a boundary separating data points, ASVs, based on the nodule phenotype of *L. burttii* plants inoculated with soil 2 suspension. The SVM model revealed that *Mesorhizobium* ASV 1 (M.1) was by far the most dominant indicator of a healthy nodule (Fig. 3), which is not surprising as *Mesorhizobium* is the typical symbiont of *L. burttii* (Rodpothong *et al.*, 2009). The second two most influential indicators of a healthy microbiome were *Pseudomonas* ASVs 28 and 57 (P.28 and P.57), which were present in both soil suspension inputs. The three ASVs most indicative of a starved *L. burttii* nodule microbiome were *Rhizobium* ASVs. Once we had identified the genera most characteristic of healthy and starved *L. burttii* nodules we wanted to predict how they interacted. A microbial network was constructed with soil suspension 2 inoculated *L. burttii* samples by using SparCC (Friedman & Alm, 2012) which analysed interactions between and within ASVs from different genera (Figs 4, S6). The ratios of negative to positive interactions within *Rhizobium* and *Mesorhizobium* ASVs were 1.13 and 1.05, respectively. *Pseudomonas* ASVs all correlated positively with one another (number of edges = 8). The ratio of negative to positive interactions between *Rhizobium* and *Mesorhizobium* (ratio = 1.64) was higher compared with this ratio among *Pseudomonas* and *Mesorhizobium* (ratio = 0.77), indicating that symbiotically beneficial *Mesorhizobium* ASVs co-occur with *Pseudomonas*. Strikingly, between *Pseudomonas* and *Rhizobium* ASVs, all correlations were negative (number of edges = 37) also supporting the SVM analysis, which showed that these ASVs were characteristic of healthy and starved *L. burttii* nodules, respectively.

Pseudomonas isolate co-colonises *Mesorhizobium* but not *Rhizobium*-induced nodules

To validate sequencing data, we inoculated *Lotus* plants with bacterial strains isolated from *Lotus* nodules (Table S1). To determine the nodule colonisation pattern of *Pseudomonas* sp. PLb11B, we co-inoculated a fluorescently tagged strain with either *Mesorhizobium* sp. Qb1E3-1, which induces effective nodules or *Rhizobium* sp. BW8-2, which induces ineffective nodules, onto *L. burttii*. Fluorescence microscopy revealed that 32.5% (14/43) of nodules induced by *Mesorhizobium* sp. Qb1E3-1 contained *Pseudomonas* sp. PLb11B. The *Pseudomonas* nodule colonisation was intracellular and was confined to particular areas of the nodule, only infecting a minority of nodule cells (Fig. 5). Conversely no plant nodules (0/22) induced by *Rhizobium* sp. BW8-2 contained *Pseudomonas* sp. PLb11B after a co-inoculation (Fig. 5).

Co-inoculation of a *Pseudomonas* isolate decreases ineffective nodulation by a *Rhizobium* but not a *Mesorhizobium* isolate in a species-specific manner

To investigate if the negative correlation between *Pseudomonas* and *Rhizobium* ASVs in nodules underlay an antagonistic interaction, *Lotus* plants were co-inoculated with nodule isolates *Pseudomonas* sp. Lb2C2 and *Rhizobium* sp. BW8-2 representing the

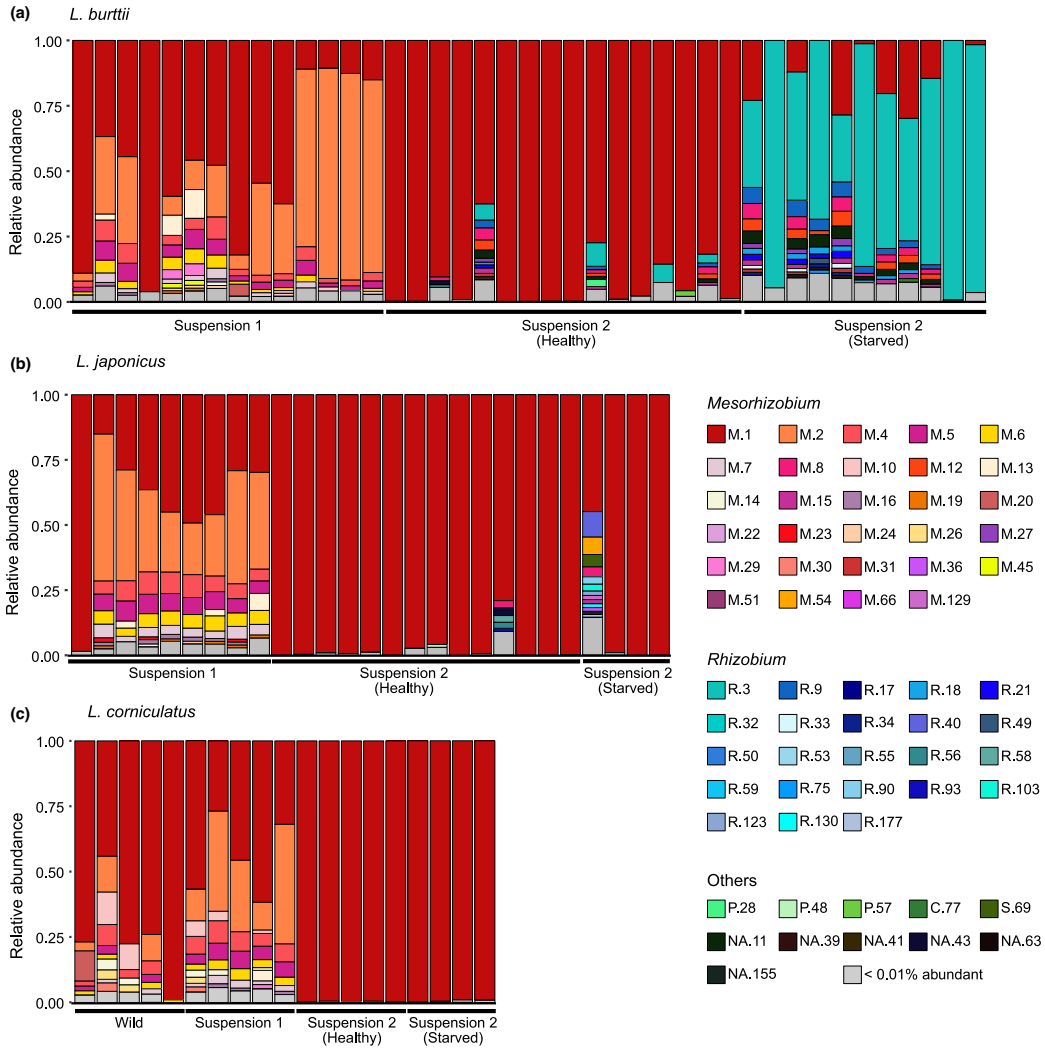

Fig. 2 Community profile showing the relative abundance of amplicon sequence variants (ASVs) present in *Lotus* nodules. The relative abundance of ASVs was estimated for *Lotus burttii* (a), *Lotus japonicus* (b) and *Lotus corniculatus* (c) using transformed data and the PHYLOSEQ v.1.26.1 package in R (McMurdie & Holmes, 2013). *Mesorhizobium* (M) ASVs are depicted in red, yellow, orange, pink and purple shades, *Rhizobium* (R) ASVs are depicted in cyan and blue shades. Other ASVs are depicted in green and black. Amplicon sequence variants < 0.01% abundant are coloured grey. NA, not assigned (taxonomy could only be defined to a Family level).

ASVs in question. *Rhizobium* sp. BW8-2 induced a large number of ineffective nodules and nodule primordia on the roots of *L. japonicus*. Co-inoculation with Lb2C2 significantly decreased the number of nodule structures (Fig. 6). No significant difference was observed regarding root weight and shoot length (Fig. S7). This inhibitory effect was host specific, as no variation in nodule number was observed in *L. burttii* upon co-inoculation with BW8-2 and Lb2C2. By contrast, co-inoculation of Lb2C2 with the effective symbiont *Mesorhizobium* sp. Qb1E3-1 saw no reduction in the nodulation of *L. burttii* or *L. japonicus* (Fig. 6) and only minimal variation in shoot length and root weight (Fig. S7). Inoculation with all three strains did not induce a

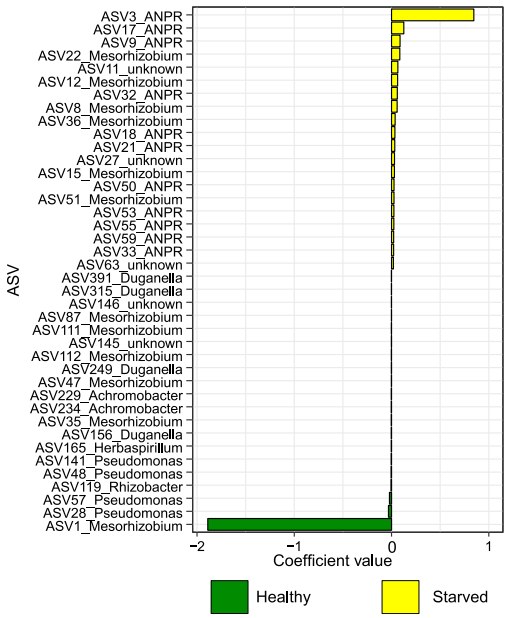

Fig. 3 Indicator amplicon sequence variants (ASVs) of samples. Support Vector Machine linear model from SCIKIT-LEARN packages were used to identify separator ASVs between healthy and starved *Lotus burttii* plants inoculated with soil 2 suspension. Histogram represents the coefficient scores of top 20 ASVs form healthy and starved plants. Negative coefficient values (green bars) represent indicator ASVs in healthy plants while positive values (yellow bars) show indicator ASVs in starved samples. The family of representative ASVs are shown after the ASV number with 'ANPR' indicating *Rhizobium* and 'unknown' indicating that no taxonomy could be assigned at a species level (NA).

different phenotype compared with co-inoculations with *Mesorhizobium* sp. Qb1E3-1 and *Rhizobium* sp. BW8-2 (Fig. S8).

Discussion

Nodules of legumes are not only colonised by rhizobia. Despite this, little information is known about how microbes other than rhizobia affect the root–nodule symbiosis, in particular nodule function and plant health. Here, we characterised variation in the bacterial microbiome of nodules dependent on plant species and soil suspension inoculum and determined correlations between the microbiome makeup and plant health using *16S* rRNA gene amplicon sequencing. Our study revealed that (1) the nodule microbiome of *L. japonicus*, *L. corniculatus* and *L. burttii* is dependent on soil suspension inoculum, (2) the nodule microbiome of starved *L. burttii* plants differs from that of the healthy plants, (3) *Pseudomonas* strains are more prevalent in healthy plant nodules than in starved-plant nodules; co-colonise effective nodules; and reduce the formation of ineffective nodules in a host-specific manner.

Soil suspension input influences *Lotus* spp. nodule microbiome

The nodule microbiome of *Lotus* plants is dependent on the soil suspension inoculum (Fig. S5; Table S3). Soil is the main influencing factor on the rhizosphere, root or nodule microbiomes in nonlegumes (Simonin *et al.*, 2020; Thiergart *et al.*, 2020) and legumes such as *M. truncatula* (Brown *et al.*, 2020) and soybean (Liu *et al.*, 2019; Han *et al.*, 2020). However, many of these studies cite the vast differences in the diversity of the microbial communities or the physicochemical properties of the soil suspension inputs as the reason for the disparity in plant microbiomes (Brown *et al.*, 2020; Han *et al.*, 2020; Simonin *et al.*, 2020). Our results showed that the nodule microbiomes of plants inoculated with different soil suspensions varied significantly (Table S3). This difference is highlighted by soil 1 suspension-inoculated nodules being colonised by a range of *Mesorhizobium* ASVs and soil 2 suspension nodules almost colonised exclusively by *Mesorhizobium* ASV M.1 (Fig. 2). Also, plants grown in soil 1 suspension produced, on average, more nodules and had a broader range of shoot growth than those inoculated with soil 2 suspension (Figs 1, S2). However, the original soil suspensions inoculated onto the plants showed no differences in alpha diversity and only slight, although not significant, differences in beta diversity (Fig. S4; Table S3). The soils from which the suspensions were produced also had no noteworthy differences in their microbiome diversity or physicochemical properties (Fig. S4; Table S2). This suggests that lowly abundant soil microbes that do not sway diversity measures, may play a pivotal role in how the microbiome functions as a whole. Such a phenomenon has been described in peat soil, where a *Desulfosporosinus* sp., which comprised only 0.006% of the total microbiome, acted as an important sulphate reducer in the biogeochemical process that diverts carbon flow from methane to CO_2 (Pester *et al.*, 2010). Also, *Bacillus* species, typically found at a low abundance in the rhizosphere compared with rhizobia, increase the number of nodules and/or the size of nodules in legumes (Rajendran *et al.*, 2008; Mishra *et al.*, 2009; Schwartz *et al.*, 2013).

Starved *L. burttii* plant nodules harbour a microbiome different to that of healthy plants

Lotus burttii is the only species that we tested that showed a significant difference between the nodule microbiome of heathy and starved plants. Nodules of starved *L. burttii* plants were dominated by *Rhizobium* ASVs, while the nodules of healthy plants were predominantly colonised by *Mesorhizobium* ASVs. *Lotus burttii* is known to form infected but ineffective nodules upon inoculation with *Rhizobium leguminosarum* Norway, however this does not form nodules on *L. japonicus* or *L. corniculatus* (Gossmann *et al.*, 2012). This correlates with the observation that starved *L. japonicus* and *L. corniculatus* harboured nodules that were not dominated by *Rhizobium*, but rather by *Mesorhizobium*

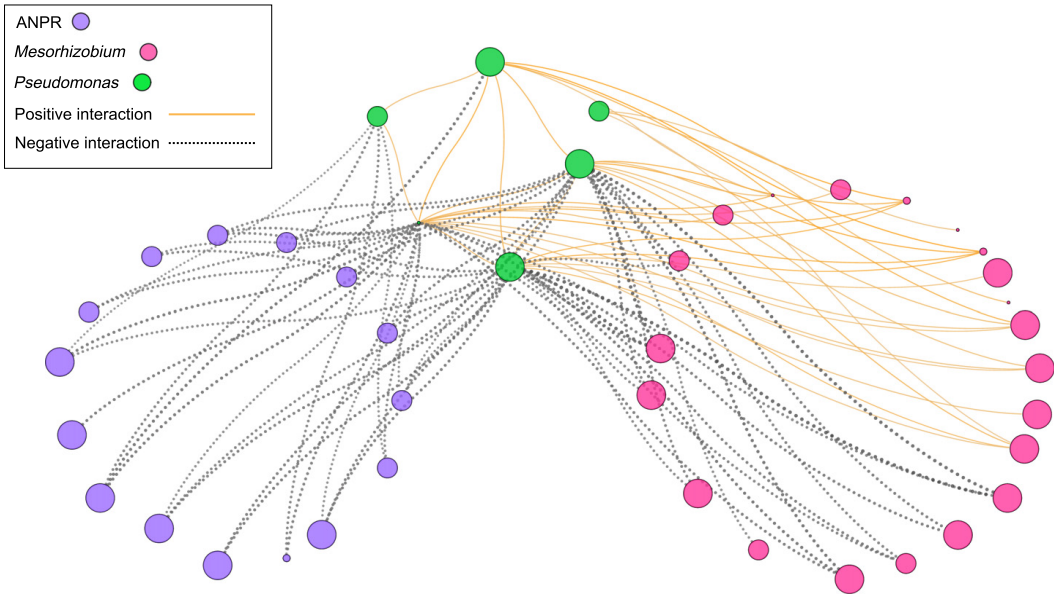

Fig. 4 Microbial co-occurrence network of *Lotus burttii*. An amplicon sequence variant (ASV) table of soil suspension 2-inoculated *L. burttii* samples was used to infer a correlation network SparCC (Friedman & Alm, 2012) algorithm implemented using the FastSpar (Watts *et al*., 2019) tool. The nodes (dots) of this network corresponding to ASVs are grouped and coloured by genus. Node size indicates the relative abundance. Each edge (line) between two ASVs represents either a positive (orange line) or negative (grey-dashed line) correlation. Only significant correlations ($|R| \geq 0.2$, $P < 0.01$) between Rhizobiaceae and Pseudomonadaceae families and first neighbours of Pseudomonadaceae are shown in the network.

ASVs, similar to the microbiome of healthy plants (Fig. 2). The variation in the starved-plant nodule microbiomes of *Lotus* species may be explained by how readily each plant is nodulated. Liang *et al.* (2019) described that ineffective *R. leguminosarum* Norway colonises nodules of *L. burttii* via cracks in the epidermis. *Lotus burttii* is more susceptible to less-specific infections (Zarrabian *et al.*, 2021), which is likely to increase its vulnerability to forming an ineffective symbiosis. This reduced specificity by *L. burttii* is also highlighted in the number of starved plants that contained nodules. In total, 73.8% of starved *L. burttii* plants grew nodules, much more than in *L. japonicus* and *L. corniculatus* (Fig. S2). The higher frequency of nodulation coupled with the reduced specificity that *L. burttii* exhibits in choosing a nodulation partner might leave the plant susceptible to expending energy on ineffective symbiotic processes, resulting in the starvation of the plant. Conversely, *L. corniculatus* and *L. japonicus* do not exhibit this same level of promiscuity, which is evidenced by their nodules being dominated by *Mesorhizobium* in all sample types. The reason as to why a starved plant would harbour a nodule microbiome similar to that of a healthy plant remains to be elucidated. We postulate that it may be simply a delay in the establishment of a successful symbiosis or due to being colonised by nonnitrogen-fixing *Mesorhizobium* strains. Rodpothong *et al.*, came to similar conclusions when inoculating *Mesorhizobium loti* Nod factor synthesis mutants onto different *Lotus* species. Nodulation of *L. burttii* was unaffected, while *L. japonicus* and *L. corniculatus* exhibited delayed nodulation and reduced infection (Rodpothong *et al.*, 2009). Taken together, our results support the idea that the reduced specificity exhibited by *L. burttii* during root–nodule symbiosis allows for a broader range of bacteria to colonise its nodules.

Pseudomonas ASVs are more prevalent in healthy *L. burttii* nodules and can reduce ineffective nodulation in *L. japonicus*

Although the microbiota of all nodule types were dominated by Rhizobiales bacteria, there was a small contingent of non-Rhizobiales ASVs detected as well (Fig. 2). This is not uncommon in legume nodules, as non-Rhizobiales bacteria are often isolated from nodules. Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Actinobacteria have all been found in various legumes nodules (Benhizia *et al.*, 2004; Dey *et al.*, 2004; Cummings *et al.*, 2009; Ibáñez *et al.*, 2009; Ampomah & Huss-Danell, 2011; Zhao *et al.*, 2013; Dobritsa & Samadpour, 2016; Ferchichi *et al.*, 2019). Of the non-rhizobia that were present in *Lotus* nodules, *Pseudomonas* was the most prevalent. We found that *Pseudomonas* ASVs were characteristic of healthy, but not of starved, *L. burttii* nodules (Fig. 3) suggesting that they have the potential to support plant health. Previous studies have shown that

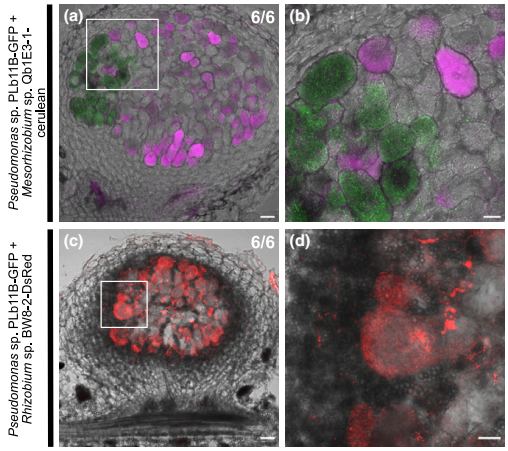

Fig. 5 *Pseudomonas* sp. PLb11B-GFP colonisation in nodules induced by *Mesorhizobium* sp. Qb1E3-1-cerulean and *Rhizobium* sp. BW8-2-DsRed. Nodules were prescreened for signs of fluorescence before sectioning. (a) Overview and (b) zoomed-in confocal microscopy image of *Pseudomonas* sp. PLb11B-GFP (green) and *Mesorhizobium* sp. Qb1E3-1-cerulean (magenta) colonisation in an effective *Lotus burttii* nodule. Overview image bar, 50 μm. Zoomed image bar, 20 μm. (c) Overview and (d) zoomed-in confocal microscopy image of *Pseudomonas* sp. PLb11B (green) and *Rhizobium* sp. BW8-2 (red) colonisation in an ineffective *L. burttii* nodule. Overview image bar, 50 μm. Zoomed image bar, 20 μm.

Pseudomonas can influence plant growth directly by producing siderophores, solubilising phosphate and producing indoleacetic acid (Dey *et al.*, 2004; Ibáñez *et al.*, 2009; Zhao *et al.*, 2013; Ferchichi *et al.*, 2019) or indirectly via antagonistic behaviour towards phytopathogenic fungi (Sindhu & Dadarwal, 2001; Chandra *et al.*, 2020). A *Pseudomonas* strain isolated from *Sophora alopecuroides* also promotes plant growth upon reinoculation with *Mesorhizobium* (Zhao *et al.*, 2013). We posit that potential microbe–microbe interactions involving *Pseudomonas* also influence the outcome of the root–nodule symbiosis. To analyse any potential microbe–microbe interactions within the nodules we looked for interactions between nodule ASVs. Network analysis comparing the nodule microbiome of healthy and starved *L. burttii* plants revealed significant negative correlations between *Pseudomonas* ASVs and multiple *Rhizobium* ASVs, as well as positive and negative interactions with *Mesorhizobium* ASVs (Fig. 4). These predicted interactions were supported by co-inoculating either an ineffective symbiont, *Rhizobium* sp. BW8-2, or an effective symbiont, *Mesorhizobium* sp. Qb1E3-1 with *Pseudomonas* sp. PLb11B. Each isolate had been previously isolated from *L. burttii* nodules, however it was found that *Pseudomonas* sp. PLb11B was only present in *Mesorhizobium*-induced nodules. Using fluorescently tagged strains and microscopy we found that 32.5% of nodules formed by the *Mesorhizobium* and 0% of nodules formed by *Rhizobium* contained *Pseudomonas*. *Pseudomonas* bacteria have been shown to colonise root hairs (Berggren *et al.*, 2005) or nodules intercellularly (Pastor-Bueis *et al.*, 2021). But in contrast, we found that *Pseudomonas* sp. PLb11B infection was intracellular and typically confined to small regions of each nodule with only a small number of cells showing extensive colonisation (Fig. 5). The lack of *Pseudomonas* sp. PLb11B in *Rhizobium* sp. BW8-2 induced nodules aligned with the sequencing data and interaction network observations. This negative interaction was further highlighted when observing another *Pseudomonas* isolate, Lb2C2, co-inoculated with *Rhizobium* sp. BW8-2 on *L. japonicus*. There was a significant reduction in the number of nodules and nodule primordia in *L. japonicus* compared with the single inoculation with the *Rhizobium* sp. BW8-2. Noticeably, this effect was host and inoculum specific, as no reduction in nodule number was observed in *L. burttii* or in co-inoculations of *Pseudomonas* with *Mesorhizobium* (Figs 6, S8). This contrasts with publications that suggested that *Pseudomonas* and *Rhizobium* strains interact synergistically (Tilak *et al.*, 2006; Egamberdieva *et al.*, 2010, 2013; Sánchez *et al.*, 2014). Interactions can be direct, for example filtrates from *Rhizobium* sp. increasing the cell density of *Pseudomonas fluorescens* (Samavat *et al.*, 2011), or mediated via the plant, for example indoleacetic acid produced by *Pseudomonas* sp. resulted in a more extensive root system in *Galega officinalis* and an increased number of potential infection sites for the compatible *Rhizobium* sp. (Egamberdieva *et al.*, 2013). The negative correlation we observed between *Pseudomonas* and *Rhizobium* ASVs in *L. burttii* nodules may also have been due to an indirect effect mediated by *Mesorhizobium*. Negative correlations were also seen between *Mesorhizobium* ASVs and *Rhizobium* ASVs. This can be explained by both bacteria competing for nodule colonisation. Significant positive correlations were apparent between *Pseudomonas* and *Mesorhizobium* ASV M.1, which was dominant in the healthy nodules of plants inoculated with soil suspension 2 (Fig. 2). Positive interactions have already been seen after the co-inoculation of *Pseudomonas* sp. isolates with a *Mesorhizobium* sp., which led to an increase in nodule number in chickpea (Malik & Sindhu, 2011). Positive correlations between *Mesorhizobium* and *Pseudomonas* coupled with the reduction in ineffective nodulation by cheater rhizobia upon co-inoculation with *Pseudomonas* supported the hypothesis that these *Pseudomonas* ASVs have a beneficial role in root–nodule symbiosis.

Our results add to the growing assertion that the soil microbiome, including non-Rhizobiales bacteria, greatly shape the overall functionality of root–nodule symbiosis and healthy plant growth (Martínez-Hidalgo & Hirsch, 2017). The ability for *Pseudomonas* to selectively colonise healthy plant nodules and reduce the number of ineffective nodules in *L. japonicus* indicated that root–nodule symbiosis is influenced by the broader soil microbiota. This research will aid the construction of synthetic communities capable of recreating observed patterns in a bid to narrow down which soil microbes and which microbe–microbe interactions are pivotal in forming the ideal microbiome to maximise plant growth.

Acknowledgements

We thank Juan Liang for help to isolate rhizobia strains, Anke Becker and Doreen Meier for providing the pABC-cerulean

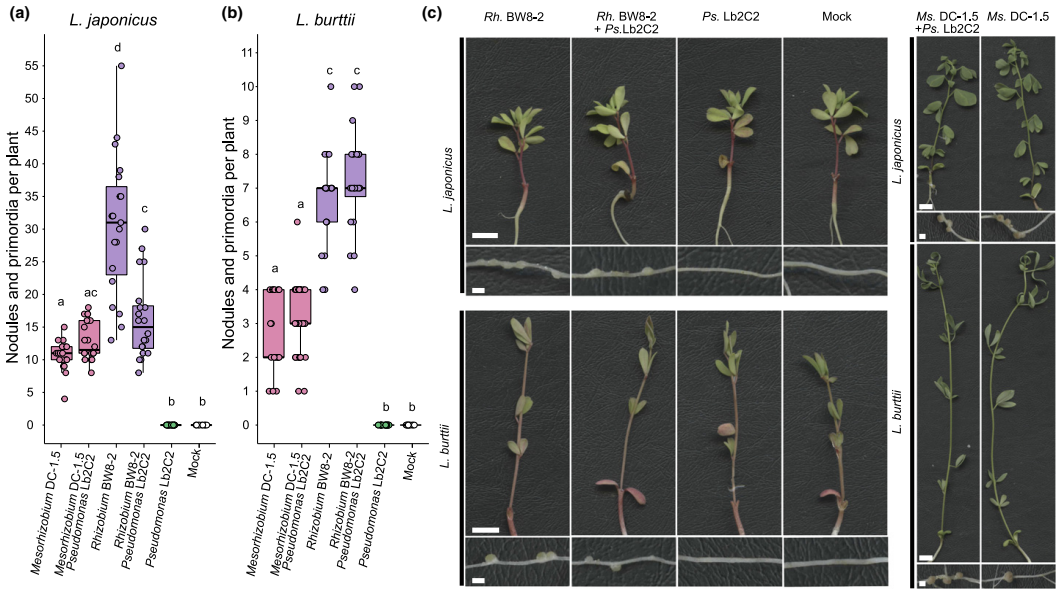

Fig. 6 Nodule organogenesis phenotype of *Lotus* plants inoculated with *Rhizobium* sp. BW8-2, *Mesorhizobium* sp. DC-1.5, and *Pseudomonas* sp. Lb2C2. Box plots of the number of nodules and nodule primordia formed on *Lotus japonicus* (a) and *Lotus burttii* (b) roots. In total, 20 plants were inoculated with *Rhizobium* sp. BW8-2, *Mesorhizobium* sp. DC-1.5 or *Pseudomonas* sp. Lb2C2 nodule isolates. *Lotus burttii* and *L. japonicus* were harvested at 4 and 5 wk post inoculation, respectively. Significance calculated using ANOVA and Tukey HSD is indicated as lowercase letters. Each point represents the number of nodules in one plant. The bold black line and the box depict the median and the interquartile range, respectively. (c) Representative images of root and shoot phenotypes of each inoculation treatment. Shoots bar, 5 mm. Nodules bar, 1 mm.

plasmid as well as Anna Beatrice Preinsberger and Oscar Gonzalez Puiggené for assisting in the construction of conjugated isolates. This work is supported by the German Research Foundation grants MA 7269/2-1 and Schl446/38-1. Open access funding enabled and organised by ProjektDEAL.

Author contributions

DBC and MMarín conceived and designed the study. DBC performed the experiments. DBC and MMahmoudi analysed the data. AB and VR performed MiSeq sequencing. EK and MS contributed with reagents and materials. DBC, MMarín and MMahmoudi contributed to writing and preparation of the manuscript. All authors read and approved the final manuscript.

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Data availability

All *16S* rRNA gene sequencing data were submitted to the Short Read Archive of NCBI and can be found under BioProject accession no. PRJNA731628. The data and scripts used for the machine learning and network analysis are available at https://github.com/IshtarMM/LotusNodules.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *Journal of Molecular Biology* **215**: 403–410.
Ampomah OY, Huss-Danell K. 2011. Genetic diversity of root nodule bacteria nodulating *Lotus corniculatus* and *Anthyllis vulneraria* in Sweden. *Systematic and Applied Microbiology* **34**: 267–275.
Bach HJ, Tomanova J, Schloter M, Munch JC. 2002. Enumeration of total bacteria and bacteria with genes for proteolytic activity in pure cultures and in environmental samples by quantitative PCR mediated amplification. *Journal of Microbiological Methods* **49**: 235–245.
Benhizia Y, Benhizia H, Benguedouar A, Muresu R, Giacomini A, Squartini A. 2004. Gamma proteobacteria can nodulate legumes of the genus *Hedysarum*. *Systematic and Applied Microbiology* **27**: 462–468.
Berggren I, Alström S, Van Vuurde J, Mårtensson AM. 2005. Rhizoplane colonisation of peas by *Rhizobium leguminosarum* bv. *viceae* and a deleterious *Pseudomonas putida*. *FEMS Microbiology Ecology* **52**: 71–78.
Bertani G. 1951. Studies on lysogenesis I: the mode of phage liberation by lysogenic *Escherichia coli*. *Journal of Bacteriology* **62**: 293–300.

Bray JR, Curtis JT. 1957. An ordination of the upland forest communities of southern Wisconsin. *Ecological Monographs* **27**: 326–349.

Brown SP, Grillo MA, Podowski JC, Heath KD. 2020. Soil origin and plant genotype structure distinct microbiome compartments in the model legume *Medicago truncatula*. *Microbiome* **8**: 1–17.

Callahan BJ, McMurdie PJ, Holmes SP. 2017. Exact sequence variants should replace operational taxonomic units in marker-gene data analysis. *The ISME Journal* **11**: 2639–2643.

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. 2016. DADA2: high-resolution sample inference from Illumina amplicon data. *Nature Methods* **13**: 581–583.

Chandra H, Kumari P, Bisht R, Prasad R, Yadav S. 2020. Plant growth promoting *Pseudomonas aeruginosa* from *Valeriana wallichii* displays antagonistic potential against three phytopathogenic fungi. *Molecular Biology Reports* **47**: 6015–6026.

Cummings SP, Gyaneshwar P, Vinuesa P, Farruggia FT, Andrews M, Humphry D, Elliott GN, Nelson A, Orr C, Pettitt D. 2009. Nodulation of *Sesbania* species by *Rhizobium* (*Agrobacterium*) strain IRBG74 and other rhizobia. *Environmental Microbiology* **11**: 2510–2525.

Cytryn E. 2013. The soil resistome: the anthropogenic, the native, and the unknown. *Soil Biology and Biochemistry* **63**: 18–23.

Dey R, Pal K, Bhatt D, Chauhan S. 2004. Growth promotion and yield enhancement of peanut (*Arachis hypogaea* L.) by application of plant growth-promoting rhizobacteria. *Microbiological Research* **159**: 371–394.

Dobritsa AP, Samadpour M. 2016. Transfer of eleven species of the genus *Burkholderia* to the genus *Paraburkholderia* and proposal of *Caballeronia* gen. nov. to accommodate twelve species of the genera *Burkholderia* and *Paraburkholderia*. *International Journal of Systematic and Evolutionary Microbiology* **66**: 2836–2846.

Dorn-In S, Bassitta R, Schwaiger K, Bauer J, Hölzel CS. 2015. Specific amplification of bacterial DNA by optimized so-called universal bacterial primers in samples rich of plant DNA. *Journal of Microbiological Methods* **113**: 50–56.

Downie JA. 2005. Legume haemoglobins: symbiotic nitrogen fixation needs bloody nodules. *Current Biology* **15**: R196–R198.

Egamberdieva D, Berg G, Lindström K, Räsänen LA. 2010. Co-inoculation of *Pseudomonas* spp. with *Rhizobium* improves growth and symbiotic performance of fodder galega (*Galega orientalis* Lam.). *European Journal of Soil Biology* **46**: 269–272.

Egamberdieva D, Berg G, Lindström K, Räsänen LA. 2013. Alleviation of salt stress of symbiotic *Galega officinalis* L. (goat's rue) by co-inoculation of *Rhizobium* with root-colonizing *Pseudomonas*. *Plant and Soil* **369**: 453–465.

Ferchichi N, Toukabri W, Boularess M, Smaoui A, Mhamdi R, Trabelsi D. 2019. Isolation, identification and plant growth promotion ability of endophytic bacteria associated with lupine root nodule grown in Tunisian soil. *Archives of Microbiology* **201**: 1333–1349.

Friedman J, Alm EJ. 2012. Inferring correlation networks from genomic survey data. *PLoS Computational Biology* **8**: e1002687.

Gamborg OL, Miller RA, Ojima K. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research* **50**: 151–158.

Gossmann JA, Markmann K, Brachmann A, Rose LE, Parniske M. 2012. Polymorphic infection and organogenesis patterns induced by a *Rhizobium leguminosarum* isolate from *Lotus* root nodules are determined by the host genotype. *New Phytologist* **196**: 561–573.

Graves S, Piepho H-P, Selzer ML. 2015. *Package 'multcompview'. Visualizations of paired comparisons*. [WWW document] URL https://cran.r-project.org/web/packages/multcompView/multcompView.pdf [accessed 14 July 2021].

Gu S, Wei Z, Shao Z, Friman V-P, Cao K, Yang T, Kramer J, Wang X, Li M, Mei X *et al.* 2020. Competition for iron drives phytopathogen control by natural rhizosphere microbiomes. *Nature Microbiology* **5**: 1002–1010.

Han Q, Ma Q, Chen Y, Tian B, Xu L, Bai Y, Chen W, Li X. 2020. Variation in rhizosphere microbial communities and its association with the symbiotic efficiency of rhizobia in soybean. *The ISME Journal* **14**: 1915–1928.

Hansen BL, Pessotti RdC, Fischer MS, Collins A, El-Hifnawi L, Liu MD, Traxler MF. 2020. Cooperation, competition, and specialized metabolism in a simplified root nodule microbiome. *mBio* **11**: e01917-20.

Herrera-Cervera JA, Caballero-Mellado J, Laguerre G, Tichy H-V, Requena N, Amarger N, Martínez-Romero E, Olivares J, Sanjuan J. 1999. At least five rhizobial species nodulate *Phaseolus vulgaris* in a Spanish soil. *FEMS Microbiology Ecology* **30**: 87–97.

Howieson JG, Yates RJ, O'Hara GW, Ryder M, Real D. 2005. The interactions of *Rhizobium leguminosarum* biovar *trifolii* in nodulation of annual and perennial *Trifolium* spp. from diverse centres of origin. *Australian Journal of Experimental Agriculture* **45**: 199–207.

Ibáñez F, Angelini J, Taurian T, Tonelli ML, Fabra A. 2009. Endophytic occupation of peanut root nodules by opportunistic *Gammaproteobacteria*. *Systematic and Applied Microbiology* **32**: 49–55.

Kelly SJ, Muszyński A, Kawaharada Y, Hubber AM, Sullivan JT, Sandal N, Carlson RW, Stougaard J, Ronson CW. 2013. Conditional requirement for exopolysaccharide in the *Mesorhizobium-Lotus* symbiosis. *Molecular Plant–Microbe Interactions* **26**: 319–329.

Kereszt A, Mergaert P, Kondorosi E. 2011. Bacteroid development in legume nodules: evolution of mutual benefit or of sacrificial victims? *Molecular Plant–Microbe Interactions* **24**: 1300–1309.

Kramina TE, Degtjareva GV, Samigullin TH, Valiejo-Roman CM, Kirkbride J, Joseph H, Volis S, Deng T, Sokoloff DD. 2016. Phylogeny of *Lotus* (Leguminosae: Loteae): partial incongruence between nrITS, nrETS and plastid markers and biogeographic implications. *Taxon* **65**: 997–1018.

Liang J, Klingl A, Lin Y-Y, Boul E, Thomas-Oates J, Marín M. 2019. A subcompatible rhizobium strain reveals infection duality in *Lotus*. *Journal of Experimental Botany* **70**: 1903–1913.

Liu F, Hewezi T, Lebeis SL, Pantalone V, Grewal PS, Staton ME. 2019. Soil indigenous microbiome and plant genotypes cooperatively modify soybean rhizosphere microbiome assembly. *BMC Microbiology* **19**: 1–19.

Malik DK, Sindhu SS. 2011. Production of indole acetic acid by *Pseudomonas* sp.: effect of coinoculation with *Mesorhizobium* sp. *Cicer* on nodulation and plant growth of chickpea (*Cicer arietinum*). *Physiology and Molecular Biology of Plants* **17**: 25–32.

Marcos-García M, Menéndez E, Cruz-González X, Velázquez E, Mateos PF, Rivas R. 2015. The high diversity of *Lotus corniculatus* endosymbionts in soils of northwest Spain. *Symbiosis* **67**: 11–20.

Martin M. 2011. CUTADAPT removes adapter sequences from high-throughput sequencing reads. *Embnet.Journal* **17**: 10–12.

Martínez-Hidalgo P, Hirsch AM. 2017. The nodule microbiome: N_2-fixing rhizobia do not live alone. *Phytobiomes* **1**: 70–82.

McMurdie PJ, Holmes S. 2013. PHYLOSEQ: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* **8**: e61217.

Mishra PK, Mishra S, Selvakumar G, Kundu S, Shankar GH. 2009. Enhanced soybean (*Glycine max* L.) plant growth and nodulation by *Bradyrhizobium japonicum-SB1* in presence of *Bacillus thuringiensis-KR1*. *Acta Agriculturae Scandinavica Section B – Soil and Plant Science* **59**: 189–196.

Noble WS. 2006. What is a support vector machine? *Nature Biotechnology* **24**: 1565–1567.

Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin P, O'hara R, Simpson G, Solymos P, Stevens MHH, Wagner H. 2018. *Community ecology package*. R package v.2.5-2. [WWW document] URL https://CRAN.R-project.org/package=vegan [accessed 22 April 2020].

Pastor-Bueis R, Jimenez-Gomez A, Barquero M, Mateos PF, González-Andrés F. 2021. Yield response of common bean to co-inoculation with *Rhizobium* and *Pseudomonas* endophytes and microscopic evidence of different colonised spaces inside the nodule. *European Journal of Agronomy* **122**: 126187.

Pedregosa F, Varoquaux G, Gramfort A, Michel V, Thirion B, Grisel O, Blondel M, Prettenhofer P, Weiss R, Dubourg V. 2011. SCIKIT-LEARN: machine learning in PYTHON. *The Journal of Machine Learning Research* **12**: 2825–2830.

Pester M, Bittner N, Deevong P, Wagner M, Loy A. 2010. A 'rare biosphere' microorganism contributes to sulfate reduction in a peatland. *The ISME Journal* **4**: 1591–1602.

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research* **41**: D590–D596.

Rajendran G, Sing F, Desai AJ, Archana G. 2008. Enhanced growth and nodulation of pigeon pea by co-inoculation of *Bacillus* strains with *Rhizobium* spp. *Bioresource Technology* **99**: 4544–4550.

Remigi P, Zhu J, Young JPW, Masson-Boivin C. 2016. Symbiosis within symbiosis: evolving nitrogen-fixing legume symbionts. *Trends in Microbiology* **24**: 63–75.

Rodpothong P, Sullivan JT, Songsrirote K, Sumpton D, Cheung KW-T, Thomas-Oates J, Radutoiu S, Stougaard J, Ronson CW. 2009. Nodulation gene mutants of *Mesorhizobium loti* R7A—*nodZ* and *nolL* mutants have host-specific phenotypes on *Lotus* spp. *Molecular Plant–Microbe Interactions* **22**: 1546–1554.

Sachs JL, Simms EL. 2008. The origins of uncooperative rhizobia. *Oikos* **117**: 961–966.

Samavat S, Besharati H, Behboudi K. 2011. Interactions of rhizobia cultural filtrates with *Pseudomonas fluorescens* on bean damping-off control. *Journal of Agricultural Science and Technology* **13**: 965–976.

Sánchez AC, Gutiérrez RT, Santana RC, Urrutia AR, Fauvart M, Michiels J, Vanderleyden J. 2014. Effects of co-inoculation of native *Rhizobium* and *Pseudomonas* strains on growth parameters and yield of two contrasting *Phaseolus vulgaris* L. genotypes under Cuban soil conditions. *European Journal of Soil Biology* **62**: 105–112.

Sandal N, Jin H, Rodriguez-Navarro DN, Temprano F, Cvitanich C, Brachmann A, Sato S, Kawaguchi M, Tabata S, Parniske M. 2012. A set of *Lotus japonicus* Gifu× *Lotus burttii* recombinant inbred lines facilitates map-based cloning and QTL mapping. *DNA Research* **19**: 317–323.

Sandman K, Ecker C. 2014. *Pseudomonas* isolation and identification: an introduction to the challenges of polyphasic taxonomy. *Journal of Microbiology & Biology Education* **15**: 287–291.

Schumpp O, Deakin WJ. 2010. How inefficient rhizobia prolong their existence within nodules. *Trends in Plant Science* **15**: 189–195.

Schwartz A, Ortiz I, Maymon M, Herbold C, Fujishige N, Vijanderan J, Villella W, Hanamoto K, Diener A, Sanders E *et al.* 2013. *Bacillus simplex*—a little known PGPB with anti-fungal activity—alters pea legume root architecture and nodule morphology when coinoculated with *Rhizobium leguminosarum* bv. *viciae*. *Agronomy* **3**: 595–620.

Seefeldt LC, Hoffman BM, Dean DR. 2009. Mechanism of Mo-dependent nitrogenase. *Annual Review of Biochemistry* **78**: 701–722.

Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T. 2003. CYTOSCAPE: a software environment for integrated models of biomolecular interaction networks. *Genome Research* **13**: 2498–2504.

Simonin M, Dasilva C, Terzi V, Ngonkeu EL, Diouf D, Kane A, Béna G, Moulin L. 2020. Influence of plant genotype and soil on the wheat rhizosphere microbiome: evidences for a core microbiome across eight African and European soils. *FEMS Microbiology Ecology* **96**: fiaa067.

Sindhu S, Dadarwal K. 2001. Chitinolytic and cellulolytic *Pseudomonas* sp. antagonistic to fungal pathogens enhances nodulation by *Mesorhizobium* sp. *Cicer* in chickpea. *Microbiological Research* **156**: 353–358.

Streeter JG. 1994. Failure of inoculant rhizobia to overcome the dominance of indigenous strains for nodule formation. *Canadian Journal of Microbiology* **40**: 513–522.

Thiergart T, Durán P, Ellis T, Vannier N, Garrido-Oter R, Kemen E, Roux F, Alonso-Blanco C, Ågren J, Schulze-Lefert P *et al.* 2020. Root microbiota assembly and adaptive differentiation among European *Arabidopsis* populations. *Nature Ecology & Evolution* **4**: 122–131.

Tilak K, Ranganayaki N, Manoharachari C. 2006. Synergistic effects of plant-growth promoting rhizobacteria and *Rhizobium* on nodulation and nitrogen fixation by pigeonpea (*Cajanus cajan*). *European Journal of Soil Science* **57**: 67–71.

Töwe S, Wallisch S, Bannert A, Fischer D, Hai B, Haesler F, Kleineidam K, Schloter M. 2011. Improved protocol for the simultaneous extraction and column-based separation of DNA and RNA from different soils. *Journal of Microbiological Methods* **84**: 406–412.

Tyc O, van den Berg M, Gerards S, van Veen JA, Raaijmakers JM, De Boer W, Garbeva P. 2014. Impact of interspecific interactions on antimicrobial activity among soil bacteria. *Frontiers in Microbiology* **5**: 567.

Venado RE, Liang J, Marín M. 2020. Rhizobia infection, a journey to the inside of plant cells. *Advances in Botanical Research* **94**: 97–118.

Vincent JM. 1970. *A manual for the practical study of the root-nodule bacteria*. Oxford, UK: Blackwell Scientific.

Wang D, Yang S, Tang F, Zhu H. 2012. Symbiosis specificity in the legume–rhizobial mutualism. *Cellular Microbiology* **14**: 334–342.

Watts SC, Ritchie SC, Inouye M, Holt KE. 2019. FASTSPAR: rapid and scalable correlation estimation for compositional data. *Bioinformatics* **35**: 1064–1066.

Werner D, Wilcockson J, Kalkowski B. 1975. Nitrogen fixing activity in separated from plant cell cultures. *Zeitschrift für Naturforschung C* **30**: 687–688.

Wickham H. 2009. Elegant graphics for data analysis. *Media* **35**: 10.1007.

William S, Feil H, Copeland A. 2012. Bacterial genomic DNA isolation using CTAB. *Sigma* **50**: 6876.

Winarno R, Lie T. 1979. Competition between *Rhizobium* strains in nodule formation: interaction between nodulating and non-nodulating strains. *Plant and Soil* **51**: 135–142.

Zarrabian M, Montiel J, Sandal N, Jin H, Lin YY, Klingl V, Marín M, James E, Parniske M, Stougaard J *et al.* 2021. A promiscuity locus confers *Lotus burttii* nodulation with rhizobia from five different genera. *bioRxiv*. doi: 10.1101/2021.08.26.457880.

Zgadzaj R, Garrido-Oter R, Jensen DB, Koprivova A, Schulze-Lefert P, Radutoiu S. 2016. Root nodule symbiosis in *Lotus japonicus* drives the establishment of distinctive rhizosphere, root, and nodule bacterial communities. *Proceedings of the National Academy of Sciences, USA* **113**: E7996–E8005.

Zhao LF, Xu YJ, Ma ZQ, Deng ZS, Shan CJ, Wei GH. 2013. Colonization and plant growth promoting characterization of endophytic *Pseudomonas chlororaphis* strain Zong1 isolated from *Sophora alopecuroides* root nodules. *Brazilian Journal of Microbiology* **44**: 629–637.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Dataset S1 Metadata of all samples.

Fig. S1 Reproducibility of plant growth experiments.

Fig. S2 Number of nodules per plant after inoculation with soil suspensions.

Fig. S3 Rarefaction curves of sequencing data.

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Fig. S5 Global principal coordinate analysis of all samples.

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Fig. S7 Root weight and shoot length phenotype of *Lotus* plants inoculated with *Rhizobium* sp. BW8-2, *Mesorhizobium* sp. DC-1.5, and *Pseudomonas* sp. Lb2C2.

Fig. S8 Nodule organogenesis phenotype of *Lotus* plants inoculated with *Rhizobium* sp. BW8-2, *Mesorhizobium* sp. DC-1.5, and *Pseudomonas* sp. Lb2C2.

Table S1 Strains and plasmids used.

Table S2 Physicochemical analysis of soil samples. h, humus soil; uL, silty clay.

Table S3 PERMANOVA analysis of beta diversity in all nodule microbiome sample types.

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***New Phytologist* Supporting information**

Microbiome profiling reveals that *Pseudomonas* antagonise parasitic nodule colonisation of cheater rhizobia in *Lotus*

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Article acceptance date: 11 January 2022

The following information is available for this article:

Table S1. Strains and plasmids used.

Strains used	Derivation and relevant genotype	Reference
Mesorhizobium sp.		
Qb1E3-1	Wild type strain isolated from a healthy *Lotus burttii* nodule, Fm^R	This work
Qb1E3-1-cerulean	*Mesorhizobium* Qb1E3-1 containing the pABC plasmid, Sp^R	This work
DC-1.5	Wild type strain isolated from a healthy *Lotus burttii* nodule	This work
Pseudomonas sp.		
Lb2C2	Wild type strain isolated from a healthy *Lotus burttii* nodule, Rif^R	This work
Lb2C2-GFP	*Pseudomonas* sp. Lb2C2 containing the pFAJ-GFP plasmid, Tc^R	This work
PLb11B	Wild type strain isolated from a healthy *Lotus burttii* nodule, Rif^R	This work
PLb11B-GFP	*Pseudomonas* sp. PLb11B containing the pFAJ-GFP plasmid, Tc^R	This work
Rhizobium sp.		
BW8-2	Wild type type strain isolated from a starved *Lotus burttii* nodule	This work
BW8-2-*Ds*Red	*Rhizobium* sp. BW8-2 containing the pFAJ-*Ds*Red plasmid, Tc^R	This work
Escherichia coli		
ST18	S17 λpir Δ*hemA*, Tp^R, Sm^R	(Thoma & Schobert, 2009)
Plasmids		
pFAJ-GFP	pFAJ1708 carries the GFP encoding gene, Tc^R	(Kelly *et al.*, 2013)
pFAJ-*Ds*Red	pFAJ1708 carries the *Ds*Red encoding gene, Tc^R	(Kelly *et al.*, 2013)
pABC-cerulean	pABC-cerulean plasmid, Tc^R	Prof. Dr. Anke Becker

Fm, fosfomycin; Sp, spectinomycin; Tc, tetracyclin; Sm, streptomycin; Rf, rifampycin; R, resistance.

Table S2. Physicochemical analysis of soil samples.

	Site 1	Site 2
Soil type	huL	uL
pH-value	7.1	7.2
P_2O_5 (mg/100g)	23	14
K_2O (mg/100g)	33	19
Mg (mg/100g)	27.8	15.3
Mn (mg/Kg)	258	136
Cu (mg/Kg)	4.1	4.1
Zn (mg/Kg)	4.3	4.3
Na (mg/Kg)	3	3
B (mg/Kg)	0.62	0.12
Fe (CAT) (mg/100g)	13.1	9.7
S (mg/Kg)	5.6	5.1
K_{fix} (mg/100g)	2	3
Org. matter %	4.7	3.5
N_{tot} %	0.27	0.2
C/N	10	10
Ca (mg/100g)	199	231
% ton	26	24
% silt	23	45
% sand	52	31

*The analysis was conducted by AGROLAB Agrarzentrum GmbH. h = humus soil; uL = silty clay.

Table S3. PERMANOVA analysis of beta diversity in all nodule microbiome sample types. The Bray-Curtis dissimilarity index (Bray & Curtis, 1957) was used to provide dissimilarity measures between the samples. PERMANOVAs were then performed with 999 permutations using the vegan V 2.5.2 package in R (Oksanen *et al.*, 2018). *** indicate p-values < 0.001. * indicate p-values < 0.05. Soil S, soil suspension.

Compared sampled types	Pr(>F)	R2	F.model	Mean Sqs	Df	Sum OfSqs
Soil S1 v Soil S2 (input suspension)	0.072	0.215	1.919	0.431	1	0.4317
Soil S1 v Soil S2 (all plant nodules)	0.001***	0.213	23.843	3.535	1	3.535
Soil S1 healthy plants - *Lb* v *Lj*	0.273	0.056	1.252	0.151	1	0.151
Soil S1 healthy plants - *Lc* v *Lj*	0.042*	0.219	4.206	0.418	1	0.418
Soil S1 healthy plants - *Lc* v *Lb*	0.1	0.116	2.635	0.286	1	0.286
Soil S2 healthy plants - *Lb* v *Lj*	0.001***	0.160	5.552	0.306	1	0.306
Soil S2 healthy plants - *Lc* v *Lj*	0.093	0.088	1.753	0.120	1	0.120
Soil S2 healthy plants - *Lc* v *Lb*	0.249	0.052	1.162	0.067	1	0.067
Lb healthy plants - soil S1 v soil S2	0.001***	0.429	21.82	1.808	1	1.808
Lj healthy plants - soil S1 v soil S2	0.002***	0.461	17.98	1.489	1	1.489
Lc healthy plants - soil S1 v soil S2	0.109	0.175	2.562	0.210	1	0.210
Lb soil S2 - healthy v starved plants	0.001***	0.525	30.95	3.055	1	3.055
Lj soil S2 - healthy v starved plants	0.097	0.051	0.869	0.061	1	0.061
Lc soil S2 - healthy v starved plants	0.742	0.050	0.428	0.025	1	0.025
Lc soil S1 - lab grown v wild plants	0.342	0.112	0.891	0.080	1	0.080
Meso S1 v *Meso* Soil 2 (input suspension)	0.41	0.128	1.028	0.162	1	0.162

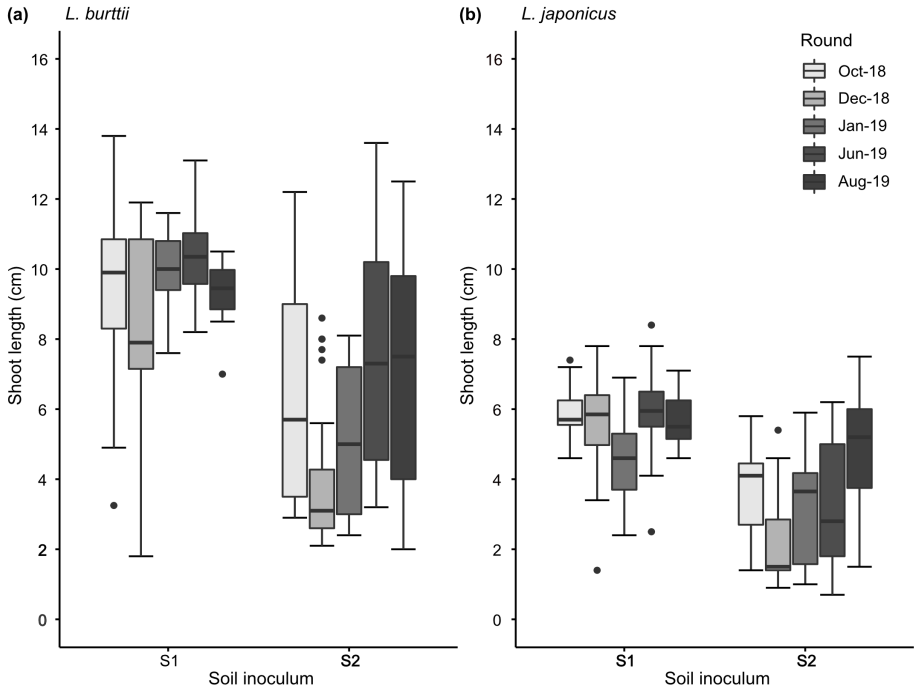

Fig S1. Reproducibility of plant growth experiments. Shoot phenotype of *L. burttii* (a) and *L. japonicus* (b) inoculated with either soil suspension 1 (S1) or soil suspension 2 (S2) from independent inoculations. Experiments were carried out in the months indicated with independent soil samples collected in October 2018 and May 2019. Between 50-100 plants per condition were grown in closed jars and harvested at 5 weeks post inoculation for each independent experiment. The bold black line and the box depict the median and the interquartile range, respectively. Black circles represent outliers.

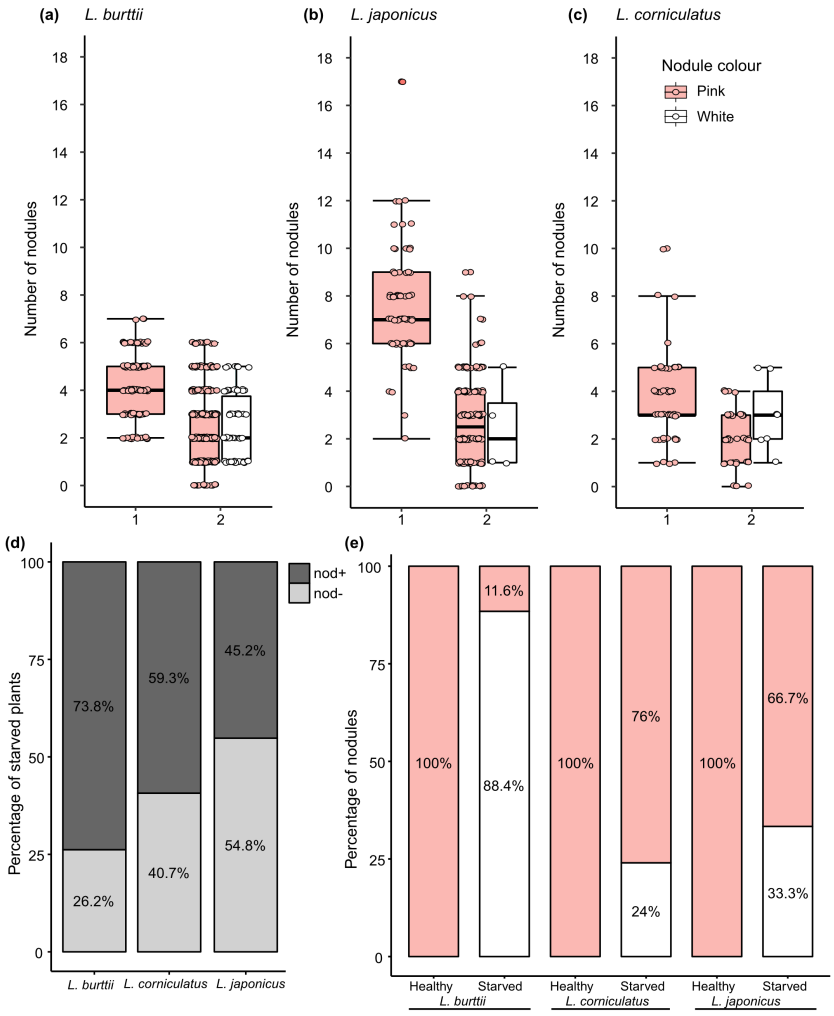

Fig S2. Number of nodules per plant after inoculation with soil suspensions. Quantification of pink and white nodules of *Lotus burttii* (a), *Lotus japonicus* (b), and *Lotus corniculatus* (c) plants grown in closed jars for 5 weeks after inoculation with soil suspensions 1 and 2. Each plot consists of results from two independent experiments. Each point represents the number of nodules in one plant. The bold black line and the box depict the median and the interquartile range, respectively. Plants that contained no nodules are not represented. Each sample type contains between 50-150 plants. (d) Percentages of starved plants of *L. burttii* (n=42), *L. corniculatus* (n=27), and *L. japonicus* (n=42) including both pink and white nodules. (e) Percentages of pink and white nodules formed according to species and phenotype after soil suspension 2 inoculation. Plants were inoculated with either soil suspension 1 or 2, grown in closed Weck jars and harvested 5 weeks post inoculation. The plot consists of results from two independent experiments.

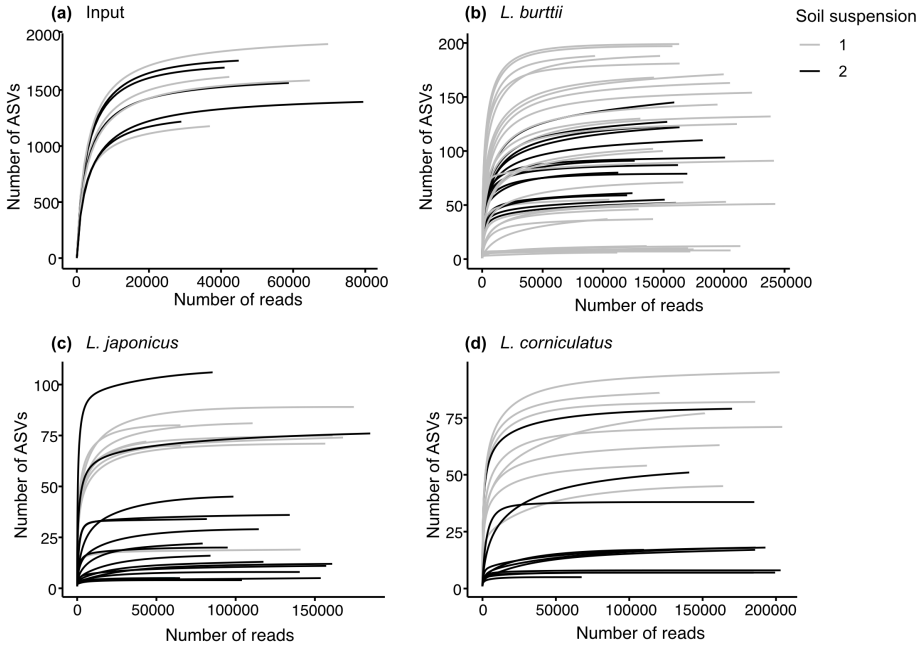

Fig S3. Rarefaction curves of sequencing data. Rarefaction curves of nodule samples from soil suspension input (a), *Lotus burttii* (b), *Lotus japonicus* (c), *Lotus corniculatus* (d) showing the number of unique amplicon sequence variants (ASVs) per total reads. Calculated using the *vegan* package in R (Oksanen *et al.*, 2018). Soil suspension input for each sample is discerned by colour.

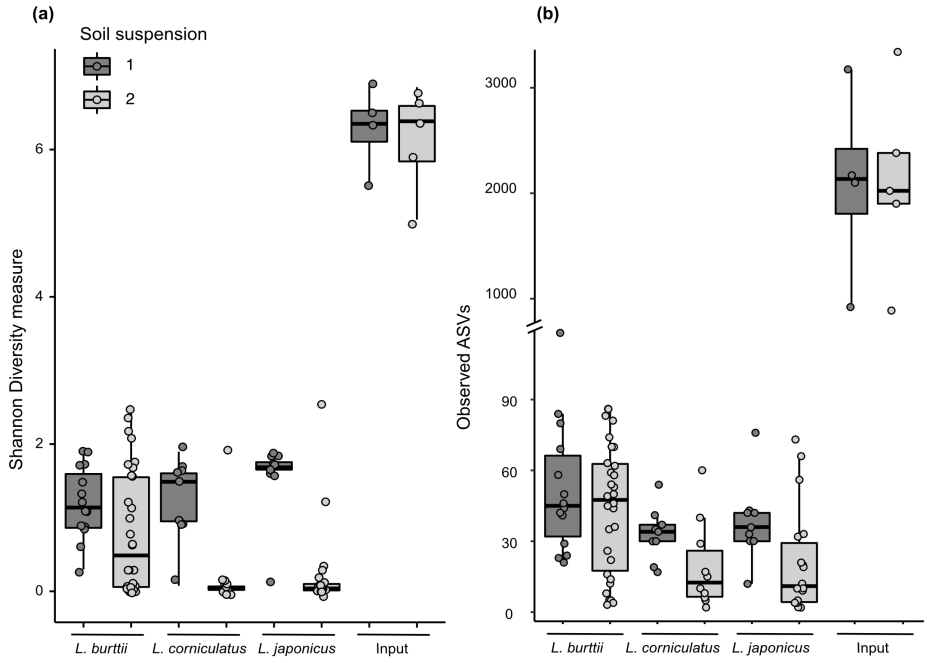

Fig S4. Nodule microbiome alpha diversity plotted by species and soil suspension input. (a) Shannon diversity measures and (b) observed amplicon sequence variants (ASVs) of all 99 samples were calculated using unfiltered data. Each point represents a nodule sample or a soil suspension input sample. The bold black line and the box depict the median and the interquartile range, respectively.

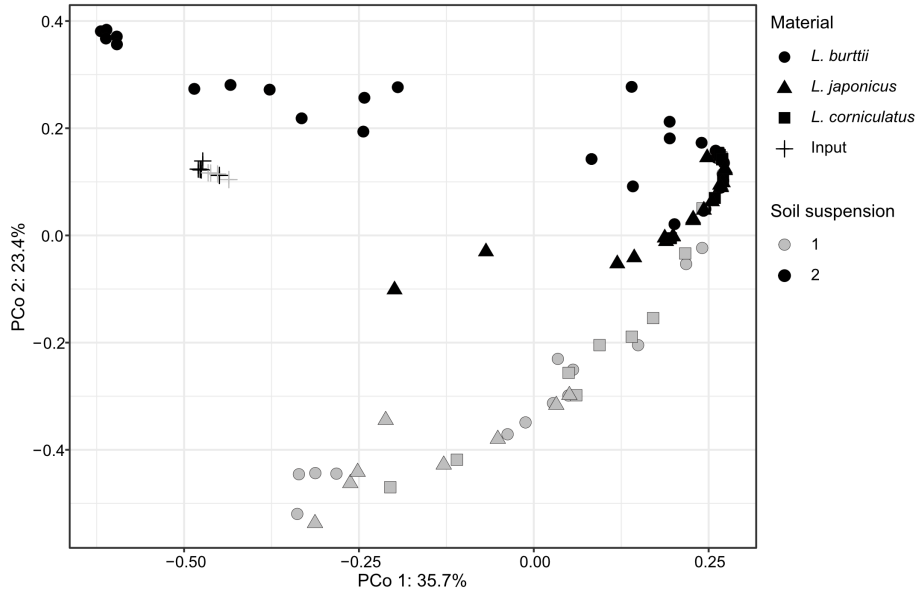

Fig S5. Global principal coordinate analysis (PCoA) of all samples. PCoA plot of all soil suspension input and nodule samples based on beta diversity calculated using the Bray-Curtis dissimilarity index (Bray & Curtis, 1957).

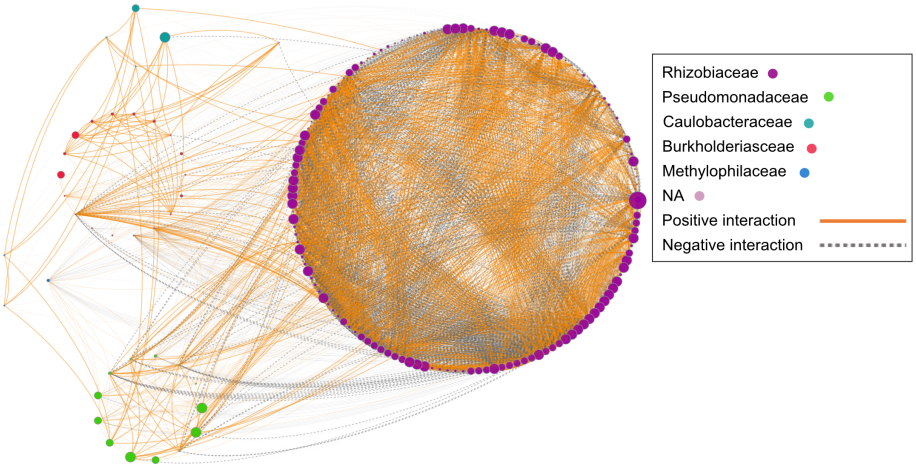

Fig S6. Overview network analysis. An amplicon sequence variant (ASV) table of *L. burttii* samples inoculated with soil suspension 2 was used to infer a correlation network using the SparCC algorithm (Friedman & Alm, 2012) implemented in FastSpar (Watts *et al.*, 2019). The nodes (dots) of this network corresponding to ASVs are grouped and coloured by Family. Node size indicates the relative abundance. Each edge (line) between two ASVs represents either a positive (orange line) or negative (grey-dashed line) correlation. Significant correlations ($|R| \geq 0.2$, $P \leq 0.01$) are shown in the network. NA indicates taxonomy of ASV could not be assigned at a family level.

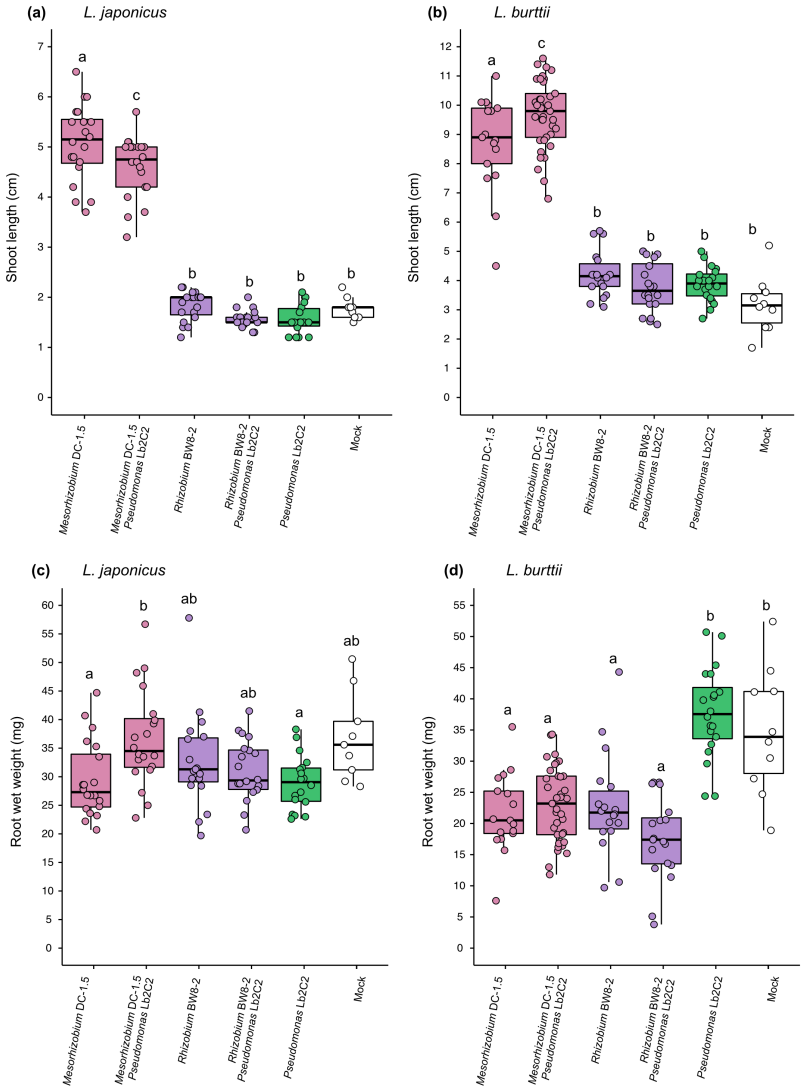

Fig S7. Root weight and shoot length phenotype of *Lotus* plants inoculated with *Rhizobium* sp. BW8-2, *Mesorhizobium* sp. DC-1.5, and *Pseudomonas* sp. Lb2C2. Box plots of the shoot length and wet root weight of *Lotus japonicus* **(a, c)** and *Lotus burttii* **(b, d)** plants. 20 plants were inoculated with *Rhizobium* BW8-2, *Mesorhizobium* sp. DC-1.5, and *Pseudomonas* Lb2C2 nodule isolates. *L. burttii* and *L. japonicus* were harvested at 4 and 5 weeks post inoculation respectively. Each point represents one plant. The bold black line and the box depict the median and the interquartile range, respectively. Significance calculated using ANOVA and Tukey HSD is indicated as lower-case letters.

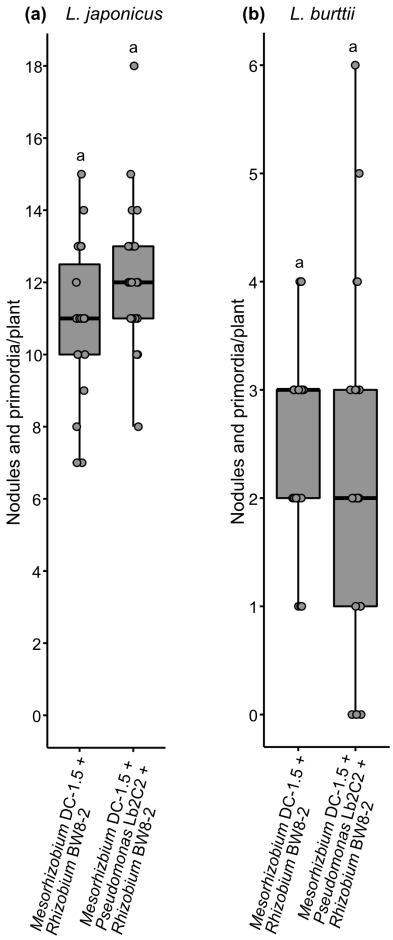

Fig S8. Nodule organogenesis phenotype of *Lotus* plants inoculated with *Rhizobium* sp. BW8-2, *Mesorhizobium* sp. DC-1.5, and *Pseudomonas* sp. Lb2C2. Box plot of the number of nodules and nodule primordia formed on *Lotus japonicus* (a) and *Lotus burttii* (b) roots. 20 plants were inoculated with *Rhizobium* sp. BW8-2, *Mesorhizobium* sp. DC-1.5, or *Pseudomonas* sp. Lb2C2 nodule isolates. *L. burttii* and *L. japonicus* were harvested at 4 and 5 weeks post inoculation, respectively. Each point represents the number of nodules in one plant. The bold black line and the box depict the median and the interquartile range, respectively. Significance calculated using ANOVA and Tukey HSD is indicated as lower-case letters.

Dataset S1. Metadata of all samples. All plants were grown in closed jars for 5 weeks. Total reads indicate the number of reads given after MiSeq sequencing. Shannon and Simpson diversity measures were calculated using the phyloseq package in R (McMurdie & Holmes, 2013). NA = not applicable. (See separate file).

References

Bach H-J, Tomanova J, Schloter M, Munch J. 2002. Enumeration of total bacteria and bacteria with genes for proteolytic activity in pure cultures and in environmental samples by quantitative PCR mediated amplification. *Journal of Microbiological Methods* **49**(3): 235-245.

Bray JR, Curtis JT. 1957. An ordination of the upland forest communities of southern Wisconsin. *Ecological Monographs* **27**(4): 326-349.

Dorn-In S, Bassitta R, Schwaiger K, Bauer J, Hölzel CS. 2015. Specific amplification of bacterial DNA by optimized so-called universal bacterial primers in samples rich of plant DNA. *Journal of Microbiological Methods* **113**: 50-56.

Friedman J, Alm EJ. 2012. Inferring correlation networks from genomic survey data. *PLoS Computational Biology* **8**: e1002687.

Herrera-Cervera JA, Caballero-Mellado J, Laguerre G, Tichy H-V, Requena N, Amarger N, Martínez-Romero E, Olivares J, Sanjuan J. 1999. At least five rhizobial species nodulate *Phaseolus vulgaris* in a Spanish soil. *FEMS Microbiology Ecology* **30**(1): 87-97.

Kelly SJ, Muszyński A, Kawaharada Y, Hubber AM, Sullivan JT, Sandal N, Carlson RW, Stougaard J, Ronson CW. 2013. Conditional requirement for exopolysaccharide in the *Mesorhizobium–Lotus* symbiosis. *Molecular Plant-Microbe Interactions* **26**(3): 319-329.

McMurdie PJ, Holmes S. 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS ONE* **8**(4): e61217.

Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin P, O'hara R, Simpson G, Solymos P, Stevens MHH, Wagner H. 2018. Community ecology package. *R package version*: 2.5-2. URL https://CRAN.R-project.org/package=vegan [accessed on 22 April 2020].

Thoma S, Schobert M. 2009. An improved *Escherichia coli* donor strain for diparental mating. *FEMS Microbiology Letters* **294**(2): 127-132.

Watts SC, Ritchie SC, Inouye M, Holt KE. 2019. FastSpar: rapid and scalable correlation estimation for compositional data. *Bioinformatics* **35**(6): 1064-1066.

Discussion

Plant nodules are colonised by symbiotic rhizobia as well as soil bacteria from other orders (Martínez-Hidalgo & Hirsch, 2017). Many of these non-rhizobia exhibit plant-health promoting traits (Tariq *et al.*, 2014; Cardoso *et al.*, 2018). Despite this, little is known about the degree to which nodule microbial assemblages can vary under certain conditions and the impact that this variation can have on the plant health. Studies into the plant-health promotion capability of nodule microbiota typically involve isolating a range of bacteria from soil inoculated or wild nodules and carrying out *in vitro* testing of potential plant growth promoting traits (Ahmad *et al.*, 2008; Selvakumar *et al.*, 2008; Fan *et al.*, 2018; Soares *et al.*, 2020). While this is a good method for identifying potentially beneficial bacteria, it does not consider the broader nodule microbial community and the interactions therein that would influence the overall function. We decided to use a more investigative approach and utilise natural variation to do comparative nodule microbiome analyses to identify potentially beneficial nodule bacteria. By identifying differences in nodule bacterial communities between variant sample types and contextualising this variation with network and supervised learning models we endeavoured to pinpoint candidates for further *in planta* experiments.

The assemblage of nodule microbiota is known to vary based on a range of external influences, both biotic and abiotic (Zhang *et al.*, 2011; Li *et al.*, 2016; Leite *et al.*, 2017; Hakim *et al.*, 2020; Mayhood & Mirza, 2021; Shah *et al.*, 2021). In this project we examined the microbiome in nodules of three *Lotus* species, inoculated with different soil suspensions and showing variant health phenotypes. The experimental procedure involved sequencing the V3-V4 regions of the 16S rRNA gene of DNA extracted from surface-sterilized nodules. Subsequent bioinformatic analyses were run to compare the variable sample types in a bid to find trends and key taxa. These results then informed synthetic inoculations comprised of nodule-isolated strains that aimed to identify the potential influence and role of the strains-of-interest in root-nodule symbiosis. The plethora of microbes that inhabit the range of plant niches available facilitates a borderline infinite number of potential interactions. For this reason, we decided to take a reductionist approach and focus exclusively on nodule endophytes. For a similar reason we also only analysed the bacterial contingent of the nodule microbiome as, compared to fungi and other microorganisms, they are the most often isolated members of nodules, even when excluding rhizobia (Martínez-Hidalgo & Hirsch, 2017).

Our results showed that (1) the microbiome of *Lotus* nodules is dependent on the soil inoculum, (2) nodule microbiome of starved *Lotus burttii* plants differ to those from healthy plants, (3) that *Pseudomonas* are more prevalent in healthy plant nodules than in starved and

(4) *Pseudomonas* sp. strain Lb2C2 reduces ineffective *Rhizobium* nodulation of *Lotus japonicus* upon co-inoculation.

1. The nodule microbiome varies between healthy *Lotus* plants inoculated with different soil suspensions

In this project we looked at the variance in nodule microbiome between plant species, soil inoculum and plant health phenotype. The soil samples used for inoculation varied in that soil 1 was taken from a site where wild *Lotus corniculatus* grew and soil 2 was taken from a site uninhabited by legumes. The most apparent phenotypic difference between soil suspension inoculation types was the variation in plant health. Soil suspension 1 inoculated plants were consistently healthy, while those inoculated with soil suspension 2 produced both healthy and starved plants (Fig. 1; Crosbie *et al.*, 2022). Abiotic factors can impact soil microbial community function, such as depth (Sokol *et al.*, 2022), tillage (Kraut-Cohen *et al.*, 2020), temperature (Adamczyk *et al.*, 2019) and moisture (Sharaf *et al.*, 2019). The most influential abiotic factor controlling bacterial communities is pH (Lauber *et al.*, 2008; Geyer *et al.*, 2014; Zhalnina *et al.*, 2015). Some bacteria have a narrow pH tolerance whereby the acidification of the cell interior compromises the function of enzymes and overall metabolism (Beales, 2004). Another reason is that pH indirectly affects bacterial community structure by altering a range of soil characteristics like nutrient availability and cationic metal solubility (Lauber *et al.*, 2009). Alterations in pH can favour certain bacteria, for example, abundance of some soil *Acidobacteria* and *Firmicutes* positively correlate with acidic pH (Zhalnina *et al.*, 2015). Before assessing which biotic influences can shape the soil microbiota, it is important to control for these abiotic influences. Therefore, we adjudged it important to use experimental methods that limit variations in abiotic factors. To this end we used a gnotobiotic system and inoculated plants with soil suspensions that contained defined pH and nutrient levels. The soil substrate was a consistent sand:vermiculite mixture and incubation was in controlled light and temperature conditions to limit microbial community variation due to abiotic factors.

Our first aim was to check whether our laboratory growth conditions were representative of those in nature. We sequenced the microbiome of wild *L. corniculatus* nodules and found that plants growing at soil site 1 did not have a statistically dissimilar nodule microbiome to those grown under laboratory conditions (Table S3; Crosbie *et al.*, 2022). We next checked if the healthy plants across both soil inoculants had a similar nodule microbiome. The first indication that there was variance in nodulation phenotype between the soil suspension inocula was the number of nodules produced. *Lotus* plants inoculated with soil suspension 1 had, on average, more nodules per plant than those inoculated with soil suspension 2 (Fig. S2; Crosbie *et al.*,

2022). Several studies have cited the presence of particular *Pseudomonas* and *Bacillus* in the rhizosphere as a reason for an increase in nodule number in *Cicer arietinum* (chickpea) and *Glycine max* (soybean) respectively, although the precise mechanism with which this occurs is yet to be elucidated (Bai *et al.*, 2003; Malik & Sindhu, 2011; Egamberdieva *et al.*, 2013).

Soil is often the main determining factor for microbial communities in nodules of *Medicago truncatula* (barrel clover) and *G. max* (Liu *et al.*, 2019; Brown *et al.*, 2020; Han *et al.*, 2020). These studies cite differences in the microbial community and physicochemical properties of the soil as the cause for the disparity seen between nodule microbial communities (Brown *et al.*, 2020; Han *et al.*, 2020). A physicochemical analysis of the soils used in this project found a minor mineral variation between them and both were classified as silty-clay, although soil site 1 contained humus (Table S2; Crosbie *et al.*, 2022). Humus facilitates drainage by loosening the soil while also retaining moisture, conditions which correlate with high bacterial diversity (Mayer *et al.*, 2019). Despite these differences the microbiome of the soil suspensions did not significantly vary in alpha diversity and there was only a slight, but insignificant, variation in beta diversity (Fig. S4; Table S3; Crosbie *et al.*, 2022). This indicates that the influence these abiotic dissimilarities did impart were not enough to restructure the bacterial community in a statistically significant way.

The lack of significant difference between soil microbial communities does not indicate that the function will be the same. Minor species that do not shift diversity measures may still have a disproportionate influence over the functionality of a microbial community (Shade *et al.*, 2014; Jousset *et al.*, 2017). For example, a *Desulfosporosinus* sp. in peat soil, which makes up 0.006% of the total microbiome, acts as an important sulphate reducer in a biochemical process that diverts carbon flow from methane to CO_2 (Pester *et al.*, 2010). Even in activating conditions the *Desulfosporosinus* sp. still remains at low abundance, although it does increase ribosomal content (Hausmann *et al.*, 2016). Scarce bacteria can also have a less specific, albeit important, role in protecting against the invasive success of pathogens (Mallon *et al.*, 2015). An increase in community diversity increases resource competition and can therefore decrease invasion potential through niche pre-emption, thus giving less abundant species a disproportionate influence (Mallon *et al.*, 2015). Bacterial diversity in soil has also been shown to correlate with the production of antifungal volatiles. The loss of less abundant species in soil microbiota can result in the loss of antifungal activity shown against the phytopathogen *Fusarium oxysporum* (Hol *et al.*, 2015). The implication being that the microbe-microbe interactions they provided were pivotal in assisting the production of the antifungal volatiles, not that they were directly responsible (Hol *et al.*, 2015). A more diverse microbial community has the potential for a greater number of unique microbe-microbe interactions. The overall

function of the microbiome may be reliant on these interactions and therefore the removal of a certain species may result in an altered function.

As well as the variation in nodule number, healthy *L. burttii* and *L. japonicus* plant nodules also had a significantly different diversity of amplicon sequence variants (ASVs) based on soil inoculum (Table S3; Crosbie *et al.*, 2022). The main variation being the number of *Mesorhizobium* ASVs. Both inoculants produced nodules dominated by *Mesorhizobium* ASVs, however those inoculated with soil suspension 1 contained an array of ASVs while the plants inoculated with soil suspension 2 were almost exclusively colonised by ASV M.1 (Fig. 2; Crosbie *et al.*, 2022). This is despite there being no significant difference in the diversity of the *Mesorhizobium* contingent of the soil suspensions (Table S3; Crosbie *et al.*, 2022). One hypothesis is that lowly abundant taxa distinct to one soil site, that do not sway diversity measures, serve a function that assists *Mesorhizobium* sp. to colonise nodules. *Mesorhizobium loti* gains a competitive advantage when nodulating *Lotus* spp. upon the introduction of ACC deaminase gene *acdS* (Conforte *et al*., 2010; Nascimento *et al*., 2012). ACC deaminase is an enzyme that degrades the ethylene precursor 1-cyclopropane-1-carboxilate (ACC) (Glick, 2005). Ethylene is produced by plants as a response to stress and interacts with other signalling molecules, however excess ethylene can trigger plant senescence and impede nodulation (Oldroyd *et al.*, 2001; Abeles *et al.*, 2012; Müller & Munné-Bosch, 2015). The breakdown of ethylene by ACC deaminase reduces the harm caused by excess ethylene (Glick, 2005). If a member of the soil site 2 microbiota, or the *Mesorhizobium* representative of ASV M.1, demonstrates ACC deaminase activity then this could favour the nodulation of a particular *Mesorhizobium* sp. that closely associates with it. Conversely the presence of ACC deaminase also leads to an increase in nodule number (Nascimento *et al.*, 2012), an observation seen in soil suspension 1 inoculated plants, perhaps indicating the presence of bacteria that produce the enzyme in this soil inoculum. Future experiments addressing the ACC deaminase production of nodule isolates will be needed to address this hypothesis.

Mesorhizobium-Mesorhizobium competition is known to vary dependent on soil conditions. *Mesorhizobium muleiense* was found to outcompete *Mesorhizobium mediterraneum* and *Mesorhizobium ciceri* for nodulation of *Cicer arietinum* L. (chickpea) when grown in its native soil. However, when grown in a non-indigenous soil or a gamma radiation-treated sterile soil the latter two *Mesorhizobium* spp. were able to outcompete *M. muleiense* (Zhang *et al.*, 2014). This indicates that successful nodulation by *M. muleiense* is also dependent on the indigenous soil organisms. Whether this is the reason for the limited number of *Mesorhizobium* ASVs

seen in nodules of soil suspension 2 inoculated *Lotus* plants will need further evidence to be validated.

The plant genotype had a lesser impact on the nodule microbial community structure compared to the soil inoculum. Similarly, *Vigna unguiculata* (cowpea) also has a nodule microbial community shaped more by soil than plant genotype (Leite *et al.*, 2017) and *M. truncatula* genotypes vary in the composition, but not in the diversity, of nodule endophytes (Burns *et al.*, 2021). The three *Lotus* species used in this project all belong to the same phylogenetic branch and the same cross-inoculation group, being effectively nodulated by *Mesorhizobium* (Degtjareva *et al.*, 2008; Gossmann *et al.*, 2012). Pairwise comparisons of healthy plants addressing distinctions due to genotype only saw a significant difference between *L. japonicus* vs *L. corniculatus* inoculated with soil suspension 1 and *L. burttii* vs *L. japonicus* inoculated with soil suspension 2 (Table S3; Crosbie *et al.*, 2022). The latter of which is likely due to the number of non-*Mesorhizobium* present in *L. burttii* soil suspension 2 samples. Most non-*Mesorhizobium* strains isolated in this project were from *L. burttii* nodules, which hints at the level of stringency that *L. burttii* imposes on potential nodule colonisers (Table S1; Crosbie *et al.*, 2022).

2. Only *Lotus burttii* showed a variation in nodule microbiome based on health of the plant

Lotus burttii was the only *Lotus* species tested that exhibited a difference in nodule microbiome between plant health phenotypes (Fig. 1e; Fig. 2; Crosbie *et al.*, 2022). Like both other species, healthy *L. burttii* nodules were found to be dominated by *Mesorhizobium* ASVs, however the starved plant nodules were mostly occupied by *Rhizobium*-like ASVs (Fig. 2; Crosbie *et al.*, 2022). The difference in starved plant nodule community between the *Lotus* species tested may be attributed to the variation in promiscuity of symbiotic partner selection. *L. burttii* is known to be a more promiscuous plant, in terms of symbiotic pairing, than the other two *Lotus* spp. tested (Rodpothong *et al.*, 2009; Zarrabian *et al.*, 2021). For example, *L. burttii* is known to form ineffective nodules with *Rhizobium leguminosarum* Norway, a strain that does not nodulate *L. corniculatus* or *L. japonicus* (Gossmann *et al.*, 2012). *L. burttii* is also able to form nodules with 5 distinct genera (Zarrabian *et al.*, 2021). The promiscuity of *L. burttii* in this project was evidenced by observing the nodulation rate of the starved plants. Of the starved *L. burttii* plants, 73.8% contained nodules, while only 59.3% and 45.2% of *L. corniculatus* and *L. japonicus* starved plants contained nodules respectively (Fig. S2d; Crosbie *et al.*, 2022). The remaining starved plants had no nodules. The ability for *L. burttii* to form

nodules with both *Mesorhizobium* sp. and *Rhizobium* sp. facilitates the variation seen between the starved and healthy plants that is not seen in the other *Lotus* species.

Another observation that sheds light on the difference between the starved plant phenotypes of *Lotus* is seen in the nodule colour. Starved *L. burttii* plants contained a much higher percentage of white nodules than starved *L. corniculatus* and *L. japonicus* plants (Fig. S2e; Crosbie *et al.*, 2022). The white colour comes from the lack of leghemoglobin, an oxygen-carrying protein important for successful nitrogen fixation (Viands *et al.*, 1979). *nifH* mutants of *Rhizobium leguminosarum* bv. viciae, that do not fix nitrogen, produce white nodules on *Pisum sativum* cv Avola (pea) indicating a lack of leghemoglobin (Westhoek *et al.*, 2017). *G. max* co-inoculated with a nitrogen-fixing *Bradyrhizobium* and a non-fixing mutant produced nodules that showed pink and white regions, indicating that the leghemoglobin localises with nitrogen-fixing bacteria (Hahn & Studer, 1986). Presumably the plant forgoes the production of leghemoglobin when a sub-optimal colonisation occurs. Whether the lack of fixation is due to the bacteria not producing nitrogenase or the plant creating an environment not conducive to effective nitrogenase function remains to be elucidated.

The comparative lack of symbiotic promiscuity that *L. corniculatus* and *L. japonicus* exhibited in this experiment is highlighted by the similarity seen between the nodule microbiomes of the healthy and starved plants (Table S3; Crosbie *et al.*, 2022). Despite the variation in plant health phenotype all nodules were predominantly occupied by *Mesorhizobium* ASVs (Fig.2b-c; Crosbie *et al.*, 2022). Whether these plants contain a *Mesorhizobium* strain that does not fix nitrogen or the plants showed a delay in nodulation, and have therefore not yet reaped the benefits of fixed nitrogen, remains unclear. One indication that the former may be the case was seen when running a support vector machine (SVM) analysis to find the ASVs typical of starved and healthy plant nodules. “SVM is a machine learning method used to separate a data set using a linear or nonlinear surface (Noble, 2006). In this instance we used a linear-kernel to transform the data and then based on this transformation defined a boundary separating data points, ASVs, based on the nodule phenotype of *L. burttii* plants inoculated with soil 2 suspension” (Crosbie *et al.*, 2022). *Rhizobium* ASVs were found to be most indicative of a starved *L. burttii* nodule, although there were also several *Mesorhizobium* ASVs that were more prevalent in starved nodules than in healthy (Fig. 3; Crosbie *et al.*, 2022). It must be noted that this analysis was just run on *L. burttii* samples, so only similarities can be drawn, not conclusions. The pink colouration of the *L. japonicus* and *L. corniculatus* nodules indicates the presence of leghemoglobin and therefore suitable conditions for nitrogenase to function. Mutants of *Mesorhizobium loti* MAFF 303099 that have an approximately 45% reduction in nitrogenase activity, as measured by acetylene reduction assay, showed a

significant decrease in shoot growth and nodule mass after inoculation on *L. japonicus* (Quides *et al*., 2017). A decrease in nitrogenase activity may explain the starved plant phenotype despite the presence of pink nodules.

The experimental design of this project necessitated that nodules be pooled together before extracting DNA. Therefore, the observation that starved *L. japonicus* and *L. corniculatus* nodules contain *Mesorhizobium* may be an indication that effective nodules were pooled with ineffective ones (Fig. 3; Crosbie *et al.*, 2022). However, these may also be symbiotically ineffective *Mesorhizobium*. This hypothesis aligns with the explanation that the lack of difference seen between starved and healthy plant nodule microbiomes of *L. corniculatus* and *L. japonicus* is due to some *Mesorhizobium* being ineffective nitrogen fixers. A similar case is seen with *Mesorhizobium opportunistum* WSM2027^{T}, a strain that has been found to nodulate *Biserrula pelecinus* but is ineffective at fixing nitrogen (Reeve *et al.*, 2013). The ineffective nature of some *Mesorhizobium* may have evolved and persisted due to plants generally being colonised by multiple lineages at once, lessening any negative impact from a cheater (Douglas, 2008; Ling *et al.*, 2013; Moyano *et al.*, 2017). An alternative hypothesis is that cheaters can accrue more resources and gain a competitive advantage through the energy saved by not fixing nitrogen (Kiers *et al.*, 2003; Ling *et al.*, 2013). Introduced *M. ciceri* has been found to genetically diversify over time, with new strains becoming more competitive, in terms of nodule occupancy, and some ineffective at fixing nitrogen in *B. pelecinus* (Nandasena *et al*., 2007). This observation that some *Mesorhizobium* can diversify into non-nitrogen-fixing strains lends credence to the hypothesis that ineffective strains are responsible for the starved phenotype of *L. corniculatus* and *L. japonicus*. The overwhelming majority of *Mesorhizobium* ASVs that colonised soil 2 plants belonged to ASV M.1. As the ASV sequence is quite short it is possible that genetic variations explaining the ineffective variant were not captured. However, the above described *M. opportunistum* had a significantly different 16S rRNA gene to those isolated from the same region and plant (Nandasena *et al.*, 2009). Alternatively, the genetic reason for the lack of effective nitrogen-fixation is not visible in the 16S rRNA gene, instead being due to the loss or mutation of genes vital to the process of nitrogen-fixation.

3. *Mesorhizobium* and *Rhizobium* nodule co-colonisation is species and strain dependent

SVM analysis revealed that *Rhizobium* ASVs were most indicative of starved *L. burttii* nodules (Fig. 3; Crosbie *et al.*, 2022). The frequency of *Rhizobium* ASVs seen in starved *L. burttii* nodules is in stark contrast to the lack of *Rhizobium* ASVs found in *L. corniculatus* and *L. japonicus* (Fig. 2; Crosbie *et al.*, 2022). Despite this, *Rhizobium* strains have been found to

co-colonise *L. corniculatus* nodules with *Mesorhizobium* (Gossmann *et al.*, 2012; Liang, 2020). For instance, *R. leguminosarum* Norway was originally isolated from *L. corniculatus* along with a novel *Mesorhizobium* strain, *Mesorhizobium norvegicum* (Gossmann *et al.*, 2012; Kabdullayeva *et al.*, 2020). Despite the inability to nodulate *L. corniculatus* alone, *R. leguminosarum* Norway was found to contribute to the root colonisation of the beneficial nodulator, *M. norvegicum*. *R. leguminosarum* Norway enhanced the distribution of the rhizobia on plant roots and also formed biofilms *in vitro* (Kabdullayeva, 2019; Liang, 2020). This benefit to the successful symbiosis of *Lotus* and *Mesorhizobium* may indicate why a strain that cannot directly benefit the plant may still colonise the nodule. The novel *M. norvegicum* was also physiologically and chemotaxonomically characterised in this project (Kabdullayeva *et al.*, 2020). *R. leguminosarum* Norway grows almost twice as fast as *M. norvegicum* therefore forming a biofilm together could aid the *Mesorhizobium* in colonising the root faster (Liang *et al.*, 2018; Kabdullayeva *et al.*, 2020).

4. *Pseudomonas* ASVs are typical of healthy *L. burttii* nodules

After we discovered that only *L. burttii* showed variation in microbiome based on plant health phenotype, we then wanted to see which ASVs were characteristic of these sample types. Many plant growth-promoting bacteria (PGPB) that benefit legume plant growth are also able to colonise nodules (Martínez-Hidalgo & Hirsch, 2017). Legume nodules have been found to contain *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria* and *Actinobacteria* (Benhizia *et al.*, 2004; Dey *et al*., 2004; Cummings *et al*., 2009; Ampomah & Huss-Danell, 2011; Zhao *et al*., 2013; Ibáñez *et al*., 2017; Ferchichi *et al*., 2019). The low abundance and inconsistent presence of particular PGPB in nodules suggests that they are not essential for a successful symbiosis (Mayhood & Mirza, 2021), however several co-inoculation experiments have exhibited a clear benefit to plant growth (Argaw, 2012; Egamberdieva *et al.*, 2013; Zhao *et al.*, 2013; Velázquez *et al.*, 2017). It is unclear how frequently non-rhizobial PGPB colonise healthy plant nodules. By using support SVM learning we found that, aside from the *Mesorhizobium* ASV M.1, two *Pseudomonas* ASVs (P.28 and P.57) were the most indicative of a healthy *L. burttii* nodule microbiome inoculated with soil suspension 2 (Fig. 3; Crosbie *et al.*, 2022). The precise role of the *Pseudomonas* inside the nodule is unclear. *Pseudomonas* are commonly found to have plant-growth promoting traits and are often co-isolated from plant nodules (Glick, 2012; Sánchez *et al.*, 2014; Kumawat *et al.*, 2019). *Pseudomonas* co-inoculation experiments on legumes with the respective symbiont have seen plant growth-promoting traits such as, among other benefits, relief from salt stress, protection from phytopathogens and the availing of nutrients (Sindhu & Dadarwal, 2001; Argaw, 2012; Egamberdieva *et al.*, 2013; Chandra *et al.*, 2020). Some *Pseudomonas* that endophytically

colonise *L. corniculatus* contain *alkB,* an alkane monooxygenase involved in hydrocarbon degradation, indicating that they play a role in lessening the negative impact of pollution (Pawlik *et al*., 2017).

Further evidence suggesting that *Pseudomonas* could contribute to plant health was obtained through a co-occurrence network analysis of ASVs in the healthy and starved plant microbiomes of *L. burttii* nodules. This showed that *Pseudomonas* ASVs had many positive significant correlations with *Mesorhizobium* ASVs (Fig. 4; Crosbie *et al.*, 2022). Synthetic inoculations with strains representative of these correlating ASVs could be conducted to uncover potential phenotypes that would add context to the observed network correlations. An issue translating *in silico* observations into inoculation experiments was encountered when discerning which nodule isolates were truly representative of the ASVs of interest. Many *Pseudomonas* isolates with distinct morphologies and 16S rRNA gene sequences aligned with ASV P.28 (data not shown), as the V3-V4 region of the 16S rRNA gene that was used for microbiome profiling does not cover all polymorphisms that distinguish the isolated strains. This makes it impossible to identify which *Pseudomonas* isolates are truly representative of the ASVs found to be significant in the SVM analysis. To discern which *Pseudomonas* isolates are most likely to be representative of ASV P.28, we will characterise the strains in terms of plant growth-promoting traits and nodule colonisation ability with the aim of narrowing the pool of isolates of interest. *Pseudomonas* sp. Lb2C2 and PLb11B isolated from healthy *L. burttii* nodules have both exhibited phosphate solubilisation ability and siderophore production (Preinsberger, 2021).

5. *Pseudomonas* sp. PLb11B colonises *L. burttii* nodules intracellularly

Over the course of this project a broad array of nodule colonising bacteria were isolated for plant inoculation experiments. We first wanted to see if the non-Rhizobiales endophytes could still colonise nodules with only the symbiont as a co-inoculation. We found that *Pseudomonas* sp. PLb11B co-colonises 32.5% of *Mesorhizobium* sp. Qb1E3-1-induced nodules on *L. burttii* (Fig. 5a-b; Crosbie *et al.*, 2022). Typical symbionts of legumes infect nodules via infection threads or cracks in the root (Sprent, 2007). Infection threads occur when the root hair encircles a bacterial symbiont and creates an invagination up the middle of the hair that the bacteria traverse. This pathway guides the bacteria to the nodule being concurrently formed. Here the bacteria then stay and begin the process of nitrogen fixation (Gage, 2004). It is estimated that 25% of bacterial symbionts can enter nodules via intercellular cracks at the root surface (Sharma *et al.*, 2020). While the entry process for symbionts is well understood, the methods with which non-rhizobia infect the nodule are less clear. Some non-rhizobia are

suspected to ‘hitchhike’ on the infection pathway of the symbiont. Pandya and colleagues reported that fluorescently tagged *Pseudomonas* could be seen traversing the infection threads of *Vigna radiata* when co-inoculated with its symbiont, *Ensifer adherens* (Pandya *et al.*, 2013). However, nodule occupancy was only confirmed via re-isolation from infected nodules, so no information was gleaned about whether the *Pseudomonas* colonised intra- or intercellularly. Confirmation of *Pseudomonas* sp. PLb11B nodule colonisation in this project was done microscopically as well as via re-isolation from surface sterilised nodules. Whether this *Mesorhizobium-Pseudomonas* co-colonisation indicates a potential microbe-microbe synergism or independent resourcefulness from the non-rhizobial endophyte is unclear.

One key piece of evidence that may shed light on the co-infection of *Pseudomonas* with *Mesorhizobium* is the localisation of these non-rhizobia once inside the nodule. Whether the bacteria can colonise nodules intracellularly or intercellularly may provide evidence as to whether they piggy-back on infection threads triggered by symbionts, indicating a potential close association, or if they infiltrate independently of the *Mesorhizobium* via crack entry. Both *Pseudomonas* sp. PLb11B and *Mesorhizobium* sp. Qb1E3-1 appear to colonise intracellularly, however they do not seem to co-colonise in the same nodule cells, with the *Pseudomonas* only colonising a low number of adjacent cells (Fig. 5a-b; Crosbie *et al.*, 2022). A similar sectioned colonisation pattern of a nodule has been reported when two rhizobia infect a plant (Hahn & Studer, 1986; Regus *et al.*, 2017), however this same pattern has not been described for non-rhizobial nodule colonisation. Intracellular nodule infection of *L. japonicus* is typically infection thread mediated (Markmann *et al.*, 2012; Montiel *et al.*, 2021) and *M. loti* also colonises *L. burttii* nodules via infection threads (Acosta-Jurado *et al.*, 2016). If the *Pseudomonas* enter nodules via the same infection threads as the *Mesorhizobium* then it would be expected that they would co-colonise the same cells, which they do not. Another possibility is that the *Pseudomonas* enter via crack entry before becoming intracellular. Some bacteria that intercellularly infect nodule primordia can proceed to an intracellular nodule colonisation via infection threads that form after invasion of the root tissue (James *et al.*, 1992; Subba-Rao *et al.*, 1995). Uncovering the mode of nodule infection by non-rhizobia will provide insights into the level of cooperation the plant provides when accommodating them. The confocal microscopy used in this project does suggest that the *Pseudomonas* infect nodules intracellularly (Fig. 5a-b; Crosbie *et al.*, 2022), however other *Pseudomonas* nodule endophytes have a different colonisation pattern. *Pseudomonas neoaurantiaca,* which exhibits plant growth-promoting characteristics and improves yield in *Phaseolus vulgaris*, colonises the intercellular space inside the nodule while the symbiont, *Rhizobium leguminosarum* bv. *Phaseoli*, colonises intracellular space (Pastor-Bueis *et al.*, 2021). Whether the nodule colonisation of these *Pseudomonas* spp. is plant influenced or

opportunistic from the bacteria also remains unclear. The inability for *Pseudomonas* to nodulate *Lotus* alone, as well as the general low abundance and inconsistent presence of particular non-rhizobial endophytes in nodules indicates that the latter may be the case (Mayhood & Mirza, 2021).

Unlike the *Mesorhizobium-Pseudomonas* co-colonisation pattern seen in *L. burttii* nodules, ineffective *Rhizobium*-induced nodules did not see the same co-colonisation. Confocal microscopy indicated that *Rhizobium* sp. BW8-2-*Ds*Red colonised nodules intracellularly, however 0% of these nodules contained a GFP signal in co-inoculations with *Pseudomonas* sp. PLb11B-GFP (Fig. 5c-d; Crosbie *et al.*, 2022). This is in congruence with the network analysis that showed only negative interactions between *Rhizobium* and *Pseudomonas* ASVs (Fig. 4; Crosbie *et al.*, 2022). Liang and colleagues described that *R. leguminosarum* Norway colonises *L. burttii* via cracks in the epidermis and then colonises cells both inter- and intracellularly without forming transcellular infection threads (Liang *et al.*, 2019). If *Pseudomonas* sp. PLb11B co-colonises with *Mesorhizobium* sp. Qb1E3-1-cerulean by hitchhiking on infection threads, then the mode of nodule-entry the *Rhizobium* use may be the limiting factor for *Pseudomonas* nodule co-colonisation. To fully elucidate the colonisation method of the *Pseudomonas*, further experiments tracking tagged bacterium are required.

6. The contrasting influence of *Pseudomonas* on *Lotus* when co-inoculated with *Mesorhizobium* or *Rhizobium*

Evidence suggesting that *Pseudomonas* could contribute to plant health was obtained through a co-occurrence network analysis of ASVs between the healthy and starved plant microbiomes of *L. burttii* nodules. This showed that *Pseudomonas* ASVs had exclusively negative significant correlations with the ineffective *Rhizobium* ASVs and a mix of positive and negative reactions with *Mesorhizobium* (Fig. 4; Crosbie *et al.*, 2022). We investigated these correlations between the ASVs of beneficial *Mesorhizobium*, ineffective nitrogen-fixing *Rhizobium*, and *Pseudomonas* by inoculating combinations of representative isolates onto *L. burttii* and *L. japonicus*. *Mesorhizobium* sp. DC-1.5 co-inoculated with *Pseudomonas* sp. Lb2C2 saw only a slight, but statistically significant, increase in *L. burttii* shoot growth and *L. japonicus* root wet weight with no increases in nodule number (Fig. 6, Fig. S7; Crosbie *et al.*, 2022). *Pseudomonas* strains isolated from nodules have been commonly described to produce indoleacetic acid, an auxin that can aid in root elongation (Khalid *et al.*, 2004; Malik & Sindhu, 2011; Zhao *et al.*, 2013), which may explain the increased growth of *L. japonicus* roots. Other studies have found a significant increase in several phenotypic traits such as pods per plant, seeds per pod and weight of seeds (Yadegari & Rahmani, 2010; Gopalakrishnan *et*

al., 2015). The plants in this experiment were harvested at 5 weeks after inoculation which meant that the plant had not reached maturity and no seed pods had formed. Therefore, changes in these parameters were not considered.

The most striking phenotype observed was in co-inoculations of the ineffective *Rhizobium* sp. BW8-2 with the *Pseudomonas* sp. Lb2C2. There was a significant decrease in ineffective nodulation events on *L. japonicus* when the *Pseudomonas* was included in the inoculation (Fig. 6; Crosbie *et al*., 2022). This observation was plant- and inoculum-specific as the same impediment to nodulation was not seen in *L. burttii* nor in the *Pseudomonas-Mesorhizobium* inoculations (Fig. 6; Crosbie *et al.*, 2022). Whether this variation in nodulation is due to a microbe-microbe interaction or a plant-mediated indirect interaction is unclear. There are many examples of *Pseudomonas* contributing to plant growth when co-inoculated with beneficial *Rhizobium* symbionts, many of which highlight antagonistic behaviour towards pathogens as a contributing factor (Sindhu & Dadarwal, 2001; Goel *et al.*, 2002; Gu *et al.*, 2020). Typically, microbe-microbe *Pseudomonas* antagonism in the rhizosphere is directed towards fungal phytopathogens. Direct antagonism is seen in pyocyanin-producing *Pseudomonas aeruginosa* TO3 that inhibit the growth of phytopathogen *Macrophomina phaseolina* whilst also increasing the nodule mass and nitrogenase activity of *Arachis hypogea* L. (Khare & Arora, 2011). Hydrolytic enzymes produced by rhizospheric *Pseudomonas* strain MRS23 have been shown to inhibit phytopathogenic fungal growth, leading to a suppression of plant disease in *Cicer arietinum* L. (Sindhu & Dadarwal, 2001; Goel *et al.*, 2002). Some *Pseudomonas* produce phenazines or rhamnolipids, which can have and antibacterial effect on *Bacillus* sp. and other bacteria (Mezaache-Aichour *et al.*, 2013; El-Sheshtawy & Doheim, 2014), however this kind of activity is yet to be conclusively found in the rhizosphere (Tapia-García *et al.*, 2020).

Another observation that supports the hypothesis that *Rhizobium* nodulation is inhibited by *Pseudomonas*, is that the starved *L. japonicus* plants from the soil suspension inoculations often had no nodules or primordia at all (Fig. S2d; Crosbie *et al.*, 2022). This suggests that there may be other soil components that contribute to achieving, not only a reduction in ineffective *L. japonicus* nodulation by *Rhizobium*, but a complete inhibition. The increased nodulation inhibition in more complex communities indicates there may be other bacteria that cumulatively add to the effect. A mixture of *Pseudomonas marginalis*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and *Acinetobacter* sp. suppressed root colonisation and nodulation of *Sinorhizobium meliloti* on *Medicago sativa* L. (Li & Alexander, 1986). The rate of growth was suggested to be the cause of the nodulation suppression as other slower growing bacteria did not have the same effect. *Pseudomonas* sp. Lb2C2 has a much faster growth rate than

Rhizobium BW8-2 so nodulation inhibition may be due to the *Pseudomonas* outcompeting the *Rhizobium* for root colonisation or nutrients. One issue with this hypothesis is that the inhibition is not seen in *L. burttii*. Therefore, if growth rate is the determining factor, then the broad promiscuity of *L. burttii* negates any obstructing effect that the *Pseudomonas* has on the *Rhizobium*.

Other forms of antagonism *Pseudomonas* exerts towards pathogens are due to the competition for resources. Many *Pseudomonas* species produce siderophores, which can sequester iron from the surrounding environment, often leading to iron becoming limiting and inhibiting the growth of other competing bacteria or phytopathogenic fungi (Sayyed *et al.*, 2013; Scott *et al.*, 2019; Gu *et al.*, 2020). Preliminary results indicate the *Pseudomonas* sp. Lb2C2 also has considerable siderophore production (Preinsberger, 2021). However, as with the growth rate hypothesis mentioned above, if siderophore activity is indeed a part of the mechanism with which *Pseudomonas* sp. Lb2C2 antagonise *Rhizobium* sp. BW8-2, then it remains unclear why the same phenotype is not seen in *L. burttii*. One hypothesis is that the plant may also influence this outcome. For example, if certain bacteria can trigger an immune response in *L. japonicus* that is not induced in *L. burttii* then this may lead to a variation in nodulation phenotype. Dissimilarity in response to bacterial effectors, proteins secreted by pathogenic bacteria into a host cell, is not uncommon between *Lotus* genotypes. *L. japonicus* and *L. burttii* have differing responses to the *Bradyrhizobium elkanii* USDA61 effector protein NopF. NopF triggers an inhibition of infection in *L. japonicus* but not in *L. burttii* (Kusakabe *et al*., 2020). Some bacteria can also induce a systemic immune response in certain plants against plant-pathogens. The flagellin polypeptide flg22 of the nodule endophyte *Pseudomonas fragi* Sneb1990 can trigger an immune response in *Solanum lycopersicum* L. (tomato) against the plant-parasitic *Meloidogyne incognita* (Wang, S *et al*., 2021). Similarly, the immune response of *S. lycopersicum* L. in response to *Botrytis cinerea* is stronger after prior inoculation with nodule endophytic strains of *Micromonospora* (Martínez-Hidalgo *et al*., 2015). To ascertain if *Pseudomonas* sp. Lb2C2 triggers an immune response in *L. japonicus* that is not triggered in *L. burttii* and that nodulation inhibition is plant-mediated, further experiments monitoring plant response are required.

7. Significance and application of this work

The use of PGPB-containing biofertilizer as an alternative to industrial fertilizers, which can be environmentally harmful, is garnering more interest as environmental health becomes more salient. Not only are biofertilizers more sustainable, it is also forecast to be significantly less expensive (Herridge, 2008). One obstacle in designing appropriate biofertilizers is that the

plant growth promotion may be plant- or bacteria-specific and therefore not universally applicable (Herrmann & Lesueur, 2013). We have demonstrated that different species of *Lotus* are not only differently susceptible to ineffective nodulation but also differently receptive to benefits from plant growth-promoting *Pseudomonas*. Other challenges that biofertilizers face comes from competition with indigenous soil bacteria as well as sub-optimal environmental conditions, both of which may compromise the plant growth promoting effects (Bashan, 1998). We therefore focused our comparative analysis on nodule microbiomes from soil suspension inoculated plants. This means that any nodule inhabiting strain significantly indicative of a particular phenotype has become so whilst in a microbially complex and competitive environment. We also found that plants grown in different soil inoculants harboured different nodule communities, illustrating that the plant holobiont will vary depending on the environment. By focusing on nodule endophytes from soil inoculated plants we aim to identify strains that can cooperate with the plant and the bacterial symbiont whilst also being competitive in a microbial niche as complex as soil. Further investigation is required to understand the mechanism behind *Pseudomonas* inhibiting ineffective nodulation. However, including bacteria that are antagonistic towards sub-optimal symbionts whilst also being synergistic with the desired symbiont is an avenue of research we deem worth pursuing in soil inoculant development.

8. Conclusion

In summary, we found that the nodule microbiome of healthy *Lotus* plants is mainly dependent on soil inoculum rather than genotype and that variations based on health phenotype are strictly species-specific. A specificity that is likely due to the difference in promiscuity that each plant shows in establishing symbiotic pairings. The explanation for starved *L. japonicus* and *L. corniculatus* having a similar nodule microbiome to that of healthy plants remains unclear, however an ineffective *Mesorhizobium* or a delay in reaping the benefits of nitrogen-fixation are hypotheses that can be further investigated.

Our results also add to the growing body of evidence that nodule colonising non-Rhizobiales bacteria can affect root-nodule symbiosis. The intracellular accommodation of *Pseudomonas* inside exclusively healthy *L. burttii* nodules suggests there might be some benefit to associating with these non-nodulating bacteria. The presence of plant-growth-promoting traits, coupled with the antagonism that strain Lb2C2 shows towards parasitic *Rhizobium* suggests that benefits for the plant may be both direct and indirect.

In conclusion the nodule has a diverse bacterial contingent that is susceptible to variation based on plant genotype, plant health and inoculum. The precise mechanism with which this this diversity emerges requires further investigation, although we propose that plant growth-promoting *Pseudomonas* play a role to mitigating sub-optimal symbiotic infections.

References

Abeles FB, Morgan PW, Saltveit Jr ME. Ethylene in plant biology. 2nd edition. California, USA: Academic press; 2012

Acosta-Jurado S, Rodríguez-Navarro D-N, Kawaharada Y, Perea JF, Gil-Serrano A, Jin H, An Q, Rodríguez-Carvajal MA, Andersen SU, Sandal N. 2016. *Sinorhizobium fredii* HH103 invades *Lotus burttii* by crack entry in a Nod Factor–and surface polysaccharide–dependent manner. *Molecular Plant-Microbe Interactions* **29**(12): 925-937.

Adamczyk M, Hagedorn F, Wipf S, Donhauser J, Vittoz P, Rixen C, Frossard A, Theurillat J-P, Frey B. 2019. The soil microbiome of Gloria Mountain summits in the Swiss Alps. *Frontiers in Microbiology* **10**: 1080.

Ahmad F, Ahmad I, Khan M. 2008. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *Microbiological Research* **163**(2): 173-181.

Amarger N. 1981. Competition for nodule formation between effective and ineffective strains of *Rhizobium meliloti*. *Soil Biology and Biochemistry* **13**(6): 475-480.

Ampomah OY, Huss-Danell K. 2011. Genetic diversity of root nodule bacteria nodulating *Lotus corniculatus* and *Anthyllis vulneraria* in Sweden. *Systematic and Applied Microbiology* **34**(4): 267-275.

Andrews M, James E, Cummings S, Zavalin A, Vinogradova L, McKenzie B. 2003. Use of nitrogen fixing bacteria inoculants as a substitute for nitrogen fertiliser for dryland graminaceous crops: progress made, mechanisms of action and future potential. *Symbiosis* **35**(1): 209-229.

Argaw A. 2012. Evaluation of co-inoculation of *Bradyrhizobium japonicum* and phosphate solubilizing *Pseudomonas* spp. effect on soybean (*Glycine max* L. Merr.) in Assossa Area. *Journal of Agricultural Science and Technology* **14**(1): 213-224.

Aulakh M, Wassmann R, Bueno C, Kreuzwieser J, Rennenberg H. 2001. Characterization of root exudates at different growth stages of ten rice (*Oryza sativa* L.) cultivars. *Plant Biology* **3**(02): 139-148.

Badri DV, Vivanco JM. 2009. Regulation and function of root exudates. *Plant, Cell & Environment* **32**(6): 666-681.

Bai Y, Mei L, Zuo W, Zhang Y, Gu C, Shan Y, Hu J, Dai Q. 2019. Response of bacterial communities in coastal mudflat saline soil to sewage sludge amendment. *Applied Soil Ecology* **144**: 107-111.

Bai Y, Zhou X, Smith DL. 2003. Enhanced soybean plant growth resulting from coinoculation of *Bacillus* strains with *Bradyrhizobium japonicum*. *Crop Science* **43**(5): 1774-1781.

Bamba M, Aoki S, Kajita T, Setoguchi H, Watano Y, Sato S, Tsuchimatsu T. 2019. Exploring genetic diversity and signatures of horizontal gene transfer in nodule bacteria associated with *Lotus japonicus* in natural environments. *Molecular Plant-Microbe Interactions* **32**(9): 1110-1120.

Banba M, Siddique A-BM, Kouchi H, Izui K, Hata S. 2001. *Lotus japonicus* forms early senescent root nodules with *Rhizobium etli*. *Molecular Plant-microbe Interactions* **14**(2): 173-180.

Bascompte J. 2009. Mutualistic networks. *Frontiers in Ecology and the Environment* **7**(8): 429-436.

Bashan Y. 1998. Inoculants of plant growth-promoting bacteria for use in agriculture. *Biotechnology Advances* **16**(4): 729-770.

Beales N. 2004. Adaptation of microorganisms to cold temperatures, weak acid preservatives, low pH, and osmotic stress: a review. *Comprehensive Reviews in Food Science and Food Safety* **3**(1): 1-20.

Benhizia Y, Benhizia H, Benguedouar A, Muresu R, Giacomini A, Squartini A. 2004. Gamma proteobacteria can nodulate legumes of the genus *Hedysarum*. *Systematic and Applied Microbiology* **27**(4): 462-468.

Berendsen RL, Pieterse CM, Bakker PA. 2012. The rhizosphere microbiome and plant health. *Trends in Plant Science* **17**(8): 478-486.

Berg G, Smalla K. 2009. Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *FEMS Microbiology Ecology* **68**(1): 1-13.

Bhattacharjee RB, Singh A, Mukhopadhyay S. 2008. Use of nitrogen-fixing bacteria as biofertiliser for non-legumes: prospects and challenges. *Applied Microbiology and Biotechnology* **80**(2): 199-209.

Bohlool B, Ladha J, Garrity D, George T. 1992. Biological nitrogen fixation for sustainable agriculture: A perspective. *Plant and Soil* **141**(1): 1-11.

Bolaños-Vásquez MC, Werner D. 1997. Effects of *Rhizobium tropici*, *R. etli*, and *R. leguminosarum* bv. phaseoli on nod gene-inducing flavonoids in root exudates of *Phaseolus vulgaris*. *Molecular Plant-Microbe Interactions* **10**(3): 339-346.

Bonkowski M. 2004. Protozoa and plant growth: the microbial loop in soil revisited. *New Phytologist* **162**(3): 617-631.

Bourion V, Heulin-Gotty K, Aubert V, Tisseyre P, Chabert-Martinello M, Pervent M, Delaitre C, Vile D, Siol M, Duc G. 2018. Co-inoculation of a pea core-collection with diverse rhizobial strains shows competitiveness for nodulation and efficiency of nitrogen fixation are distinct traits in the interaction. *Frontiers in Plant Science* **8**: 2249.

Brant JB, Myrold DD, Sulzman EW. 2006. Root controls on soil microbial community structure in forest soils. *Oecologia* **148**(4): 650-659.

Brightling J. 2018. Ammonia and the fertiliser industry: The development of ammonia at Billingham. *Johnson Matthey Technology Review* **62**(1): 32-47.

Brown SP, Grillo MA, Podowski JC, Heath KD. 2020. Soil origin and plant genotype structure distinct microbiome compartments in the model legume *Medicago truncatula*. *Microbiome* **8**(1): 1-17.

Bulgarelli D, Rott M, Schlaeppi K, Ver Loren van Themaat E, Ahmadinejad N, Assenza F, Rauf P, Huettel B, Reinhardt R, Schmelzer E. 2012. Revealing structure and assembly cues for *Arabidopsis* root-inhabiting bacterial microbiota. *Nature* **488**(7409): 91-95.

Burns M, Epstein B, Burghardt LT. 2021. Comparison of nodule endophyte composition, diversity, and gene content between *Medicago truncatula* genotypes. *Phytobiomes Journal* **5**(4): 400-407.

Cao H, Chen R, Wang L, Jiang L, Yang F, Zheng S, Wang G, Lin X. 2016. Soil pH, total phosphorus, climate and distance are the major factors influencing microbial activity at a regional spatial scale. *Scientific Reports* **6**(1): 1-10.

Cardoso P, Alves A, Silveira P, Sá C, Fidalgo C, Freitas R, Figueira E. 2018. Bacteria from nodules of wild legume species: Phylogenetic diversity, plant growth promotion abilities and osmotolerance. *Science of the Total Environment* **645**: 1094-1102.

Chandra H, Kumari P, Bisht R, Prasad R, Yadav S. 2020. Plant growth promoting *Pseudomonas aeruginosa* from *Valeriana wallichii* displays antagonistic potential against three phytopathogenic fungi. *Molecular Biology Reports* **47**(8): 6015-6026.

Chaparro JM, Badri DV, Vivanco JM. 2014. Rhizosphere microbiome assemblage is affected by plant development. *The ISME Journal* **8**(4): 790-803.

Chave M, Dabert P, Brun R, Godon J-J, Poncet C. 2008. Dynamics of rhizoplane bacterial communities subjected to physicochemical treatments in hydroponic crops. *Crop Protection* **27**(3-5): 418-426.

Chen L, Shi H, Heng J, Wang D, Bian K. 2019. Antimicrobial, plant growth-promoting and genomic properties of the peanut endophyte *Bacillus velezensis* LDO2. *Microbiological Research* **218**: 41-48.

Chen S, Waghmode TR, Sun R, Kuramae EE, Hu C, Liu B. 2019. Root-associated microbiomes of wheat under the combined effect of plant development and nitrogen fertilization. *Microbiome* **7**(1): 1-13.

Collins M, Thies J, Abbott L. 2002. Diversity and symbiotic effectiveness of *Rhizobium leguminosarum* bv. trifolii isolates from pasture soils in south-western Australia. *Soil Research* **40**(8): 1319-1329.

Compant S, Cambon MC, Vacher C, Mitter B, Samad A, Sessitsch A. 2021. The plant endosphere world–bacterial life within plants. *Environmental Microbiology* **23**(4): 1812-1829.

Conforte VP, Echeverria M, Sánchez C, Ugalde RA, Menéndez AB, Lepek VC. 2010. Engineered ACC deaminase-expressing free-living cells of *Mesorhizobium loti* show increased nodulation efficiency and competitiveness on *Lotus* spp. *The Journal of General and Applied Microbiology* **56**(4): 331-338.

Cordovez V, Dini-Andreote F, Carrión VJ, Raaijmakers JM. 2019. Ecology and evolution of plant microbiomes. *Annual Review Microbiology* **73**(1): 69-88.

Crews TE, Peoples M. 2004. Legume versus fertilizer sources of nitrogen: ecological tradeoffs and human needs. *Agriculture, Ecosystems & Environment* **102**(3): 279-297.

Crosbie DB, Mahmoudi M, Radl V, Brachmann A, Schloter M, Kemen E, Marín M. 2022. Microbiome profiling reveals that *Pseudomonas* antagonises parasitic nodule colonisation of cheater rhizobia in Lotus. *New Phytologist* **234**(1): 242-255.

Cummings SP, Gyaneshwar P, Vinuesa P, Farruggia FT, Andrews M, Humphry D, Elliott GN, Nelson A, Orr C, Pettitt D. 2009. Nodulation of *Sesbania* species by *Rhizobium* (*Agrobacterium*) strain IRBG74 and other rhizobia. *Environmental Microbiology* **11**(10): 2510-2525.

Currier WW, Strobel GA. 1976. Chemotaxis of *Rhizobium* spp. to plant root exudates. *Plant Physiology* **57**(5): 820-823.

Custódio V, Gonin M, Stabl G, Bakhoum N, Oliveira MM, Gutjahr C, Castrillo G. 2022. Sculpting the soil microbiota. *The Plant Journal* **109**(3): 508-522.

D'haeze W, Holsters M. 2002. Nod factor structures, responses, and perception during initiation of nodule development. *Glycobiology* **12**(6): 79R-105R.

Dawwam G, Elbeltagy A, Emara H, Abbas I, Hassan M. 2013. Beneficial effect of plant growth promoting bacteria isolated from the roots of potato plant. *Annals of Agricultural Sciences* **58**(2): 195-201.

De Bruijn FJ 2015. Biological nitrogen fixation. *Principles of Plant-microbe Interactions*: Springer, 215-224.

de Lajudie P, Huguet T. 1988. Plant gene expression during effective and ineffective nodule development of the tropical stem-nodulated legume *Sesbania rostrata*. *Plant Molecular Biology* **10**(6): 537-548.

de Ridder-Duine AS, Kowalchuk GA, Gunnewiek PJK, Smant W, van Veen JA, de Boer W. 2005. Rhizosphere bacterial community composition in natural stands of *Carex arenaria* (sand sedge) is determined by bulk soil community composition. *Soil Biology and Biochemistry* **37**(2): 349-357.

Debellé F, Moulin L, Mangin B, Dénarié J, Boivin C. 2001. Nod genes and Nod signals and the evolution of the *Rhizobium* legume symbiosis. *Acta Biochimica Polonica* **48**(2): 359-365.

Degtjareva GV, Kramina TE, Sokoloff DD, Samigullin TH, Sandral G, Valiejo-Roman CM. 2008. New data on nrITS phylogeny of *Lotus* (*Leguminosae, Loteae*). *Wulfenia* **15**: 35-49.

Denison RF. 2000. Legume sanctions and the evolution of symbiotic cooperation by rhizobia. *The American Naturalist* **156**(6): 567-576.

Dey R, Pal K, Bhatt D, Chauhan S. 2004. Growth promotion and yield enhancement of peanut (*Arachis hypogaea* L.) by application of plant growth-promoting rhizobacteria. *Microbiological Research* **159**(4): 371-394.

Ding H, Hynes MF. 2009. Plasmid transfer systems in the rhizobia. *Canadian Journal of Microbiology* **55**(8): 917-927.

Douglas AE. 2008. Conflict, cheats and the persistence of symbioses. *New Phytologist* **177**(4): 849-858.

Downie JA. 2014. Legume nodulation. *Current Biology* **24**(5): R184-R190.

Duca D, Lorv J, Patten CL, Rose D, Glick BR. 2014. Indole-3-acetic acid in plant–microbe interactions. *Antonie van Leeuwenhoek* **106**(1): 85-125.

Edmonds JW, Sackett JD, Lomprey H, Hudson HL, Moser DP. 2020. The aeroponic rhizosphere microbiome: community dynamics in early succession suggest strong selectional forces. *Antonie van Leeuwenhoek* **113**(1): 83-99.

Egamberdieva D, Berg G, Lindström K, Räsänen LA. 2010. Co-inoculation of *Pseudomonas* spp. with *Rhizobium* improves growth and symbiotic performance of fodder galega (*Galega orientalis* Lam.). *European Journal of Soil Biology* **46**(3-4): 269-272.

Egamberdieva D, Berg G, Lindström K, Räsänen LA. 2013. Alleviation of salt stress of symbiotic *Galega officinalis* L.(goat's rue) by co-inoculation of *Rhizobium* with root-colonizing *Pseudomonas*. *Plant and Soil* **369**(1): 453-465.

El-Sheshtawy H, Doheim M. 2014. Selection of *Pseudomonas aeruginosa* for biosurfactant production and studies of its antimicrobial activity. *Egyptian Journal of Petroleum* **23**(1): 1-6.

el Zahar Haichar F, Santaella C, Heulin T, Achouak W. 2014. Root exudates mediated interactions belowground. *Soil Biology and Biochemistry* **77**: 69-80.

Evans J, McNeill A, Unkovich M, Fettell N, Heenan D. 2001. Net nitrogen balances for cool-season grain legume crops and contributions to wheat nitrogen uptake: a review. *Australian Journal of Experimental Agriculture* **41**(3): 347-359.

Fan M, Liu Z, Nan L, Wang E, Chen W, Lin Y, Wei G. 2018. Isolation, characterization, and selection of heavy metal-resistant and plant growth-promoting endophytic bacteria from root nodules of *Robinia pseudoacacia* in a Pb/Zn mining area. *Microbiological Research* **217**: 51-59.

Ferchichi N, Toukabri W, Boularess M, Smaoui A, Mhamdi R, Trabelsi D. 2019. Isolation, identification and plant growth promotion ability of endophytic bacteria associated with lupine root nodule grown in Tunisian soil. *Archives of Microbiology* **201**(10): 1333-1349.

Fourie-Basson W. 2013. Golden Wattle's promiscuous relationships ensure invasion success. *Quest* **9**(3): 16-19.

Gage DJ. 2004. Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. *Microbiology and Molecular Biology Reviews* **68**(2): 280-300.

Garbaye J. 1994. Mycorrhization helper bacteria: a new dimension to the mycorrhizal symbiosis. *The New Phytologist* **128**(2): 197-210.

Geyer KM, Altrichter AE, Takacs-Vesbach CD, Van Horn DJ, Gooseff MN, Barrett JE. 2014. Bacterial community composition of divergent soil habitats in a polar desert. *FEMS Microbiology Ecology* **89**(2): 490-494.

Glick BR. 2005. Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. *FEMS Microbiology Letters* **251**(1): 1-7.

Glick BR. 2012. Plant growth-promoting bacteria: mechanisms and applications. *Scientifica* **2012**: 1-15.

Goel A, Sindhu S, Dadarwal K. 2002. Stimulation of nodulation and plant growth of chickpea (*Cicer arietinum* L.) by *Pseudomonas* spp. antagonistic to fungal pathogens. *Biology and Fertility of Soils* **36**(6): 391-396.

Good BH, McDonald MJ, Barrick JE, Lenski RE, Desai MM. 2017. The dynamics of molecular evolution over 60,000 generations. *Nature* **551**(7678): 45-50.

Gopalakrishnan S, Srinivas V, Prakash B, Sathya A, Vijayabharathi R. 2015. Plant growth-promoting traits of *Pseudomonas geniculata* isolated from chickpea nodules. *3 Biotech* **5**(5): 653-661.

Gossmann JA, Markmann K, Brachmann A, Rose LE, Parniske M. 2012. Polymorphic infection and organogenesis patterns induced by a *Rhizobium leguminosarum* isolate from *Lotus* root nodules are determined by the host genotype. *New Phytologist* **196**(2): 561-573.

Gottel NR, Castro HF, Kerley M, Yang Z, Pelletier DA, Podar M, Karpinets T, Uberbacher E, Tuskan GA, Vilgalys R. 2011. Distinct microbial communities within the endosphere and rhizosphere of *Populus deltoides* roots across contrasting soil types. *Applied and Environmental Microbiology* **77**(17): 5934-5944.

Griffiths B, Hallett P, Kuan H, Gregory A, Watts C, Whitmore A. 2008. Functional resilience of soil microbial communities depends on both soil structure and microbial community composition. *Biology and Fertility of Soils* **44**(5): 745-754.

Gu S, Wei Z, Shao Z, Friman V-P, Cao K, Yang T, Kramer J, Wang X, Li M, Mei X. 2020. Competition for iron drives phytopathogen control by natural rhizosphere microbiomes. *Nature Microbiology* **5**(8): 1002-1010.

Guo W, Zhang K, Liang Z, Zou R, Xu Q. 2019. Electrochemical nitrogen fixation and utilization: theories, advanced catalyst materials and system design. *Chemical Society Reviews* **48**(24): 5658-5716.

Gwata E, Wofford D, Pfahler P, Boote K. 2004. Genetics of promiscuous nodulation in soybean: nodule dry weight and leaf color score. *Journal of Heredity* **95**(2): 154-157.

Hahn M, Studer D. 1986. Competitiveness of a nif− *Bradyrhizobium japonicum* mutant against the wild-type strain. *FEMS Microbiology Letters* **33**(1): 143-148.

Haichar FeZ, Marol C, Berge O, Rangel-Castro JI, Prosser JI, Balesdent Jm, Heulin T, Achouak W. 2008. Plant host habitat and root exudates shape soil bacterial community structure. *The ISME Journal* **2**(12): 1221-1230.

Hakim S, Mirza BS, Imran A, Zaheer A, Yasmin S, Mubeen F, Mclean JE, Mirza MS. 2020. Illumina sequencing of 16S rRNA tag shows disparity in rhizobial and non-rhizobial diversity associated with root nodules of mung bean (*Vigna radiata* L.) growing in different habitats in Pakistan. *Microbiological Research* **231**: 126356.

Hallmann J, Berg G. 2006. Spectrum and population dynamics of bacterial root endophytes. *Microbial Root Endophytes*: **9:** 15-31.

Han Q, Ma Q, Chen Y, Tian B, Xu L, Bai Y, Chen W, Li X. 2020. Variation in rhizosphere microbial communities and its association with the symbiotic efficiency of rhizobia in soybean. *The ISME Journal* **14**(8): 1915-1928.

Hansen BL, Pessotti RdC, Fischer MS, Collins A, El-Hifnawi L, Liu MD, Traxler MF. 2020. Cooperation, competition, and specialized metabolism in a simplified root nodule microbiome. *Mbio* **11**(4): e01917-01920.

Hartman K, Tringe SG. 2019. Interactions between plants and soil shaping the root microbiome under abiotic stress. *Biochemical Journal* **476**(19): 2705-2724.

Haskett TL, Paramasivan P, Mendes MD, Green P, Geddes BA, Knights HE, Jorrin B, Ryu M-H, Brett P, Voigt CA. 2022. Engineered plant control of associative nitrogen fixation. *Proceedings of the National Academy of Sciences* **119**(16): e2117465119.

Hassani M, Durán P, Hacquard S. 2018. Microbial interactions within the plant holobiont. *Microbiome* **6**(1): 1-17.

Hausmann B, Knorr K-H, Schreck K, Tringe SG, Glavina del Rio T, Loy A, Pester M. 2016. Consortia of low-abundance bacteria drive sulfate reduction-dependent degradation of fermentation products in peat soil microcosms. *The ISME Journal* **10**(10): 2365-2375.

Heinrich W Scherer "fertilizers" in Ullmann's encyclopedia of industrial chemistry. 1st edition. Weinheim, Germany: Wiley-VCH. 2000.

Herridge D. Nitrogen-fixing leguminous symbioses: Inoculation technology for legumes. NSW, Australia: Springer. 77-115. 2008.

Herrmann L, Lesueur D. 2013. Challenges of formulation and quality of biofertilizers for successful inoculation. *Applied microbiology and biotechnology* **97**(20): 8859-8873.

Hillel D. Soil in the environment: crucible of terrestrial life. 1st edition. California, USA: Elsevier. 2007.

Ho MD, Rosas JC, Brown KM, Lynch JP. 2005. Root architectural tradeoffs for water and phosphorus acquisition. *Functional Plant Biology* **32**(8): 737-748.

Hol WG, Garbeva P, Hordijk C, Hundscheid MP, Gunnewiek PJK, Van Agtmaal M, Kuramae EE, De Boer W. 2015. Non-random species loss in bacterial communities reduces antifungal volatile production. *Ecology* **96**(8): 2042-2048.

Hounshell D. From the American system to mass production, 1800-1932: The development of manufacturing technology in the United States. 1st edition. Maryland, USA: JHU Press. 1985.

Huang X, Liu S, Liu X, Zhang S, Li L, Zhao H, Zhao J, Zhang J, Cai Z. 2020. Plant pathological condition is associated with fungal community succession triggered by root exudates in the plant-soil system. *Soil Biology and Biochemistry* **151**: 108046.

Ibáñez F, Wall L, Fabra A. 2017. Starting points in plant-bacteria nitrogen-fixing symbioses: intercellular invasion of the roots. *Journal of Experimental Botany* **68**(8): 1905-1918.

James E, Sprent J, Sutherland J, McInroy S, Minchin F. 1992. The structure of nitrogen fixing root nodules on the aquatic mimosoid legume *Neptunia plena*. *Annals of Botany* **69**(2): 173-180.

Jones KM, Sharopova N, Lohar DP, Zhang JQ, VandenBosch KA, Walker GC. 2008. Differential response of the plant *Medicago truncatula* to its symbiont *Sinorhizobium meliloti* or an exopolysaccharide-deficient mutant. *Proceedings of the National Academy of Sciences* **105**(2): 704-709.

Jousset A, Bienhold C, Chatzinotas A, Gallien L, Gobet A, Kurm V, Küsel K, Rillig MC, Rivett DW, Salles JF. 2017. Where less may be more: how the rare biosphere pulls ecosystems strings. *The ISME Journal* **11**(4): 853-862.

Kabdullayeva T. 2019. *Root Nodule Symbiosis: Interspecific synergistic interaction between Rhizobia.* Masters, Ludwig-Maximilians University.

Kabdullayeva T, Crosbie DB, Marín M. 2020. *Mesorhizobium norvegicum* sp. nov., a rhizobium isolated from a *Lotus corniculatus* root nodule in Norway. *International Journal of Systematic and Evolutionary Microbiology* **70**(1): 388-396.

Keet J-H, Ellis AG, Hui C, Le Roux JJ. 2017. Legume–rhizobium symbiotic promiscuity and effectiveness do not affect plant invasiveness. *Annals of Botany* **119**(8): 1319-1331.

Kereszt A, Mergaert P, Kondorosi E. 2011. Bacteroid development in legume nodules: evolution of mutual benefit or of sacrificial victims? *Molecular plant-microbe interactions* **24**(11): 1300-1309.

Khalid A, Arshad M, Zahir Z. 2004. Screening plant growth-promoting rhizobacteria for improving growth and yield of wheat. *Journal of Applied Microbiology* **96**(3): 473-480.

Khan MS, Zaidi A, Wani PA. 2009. Role of phosphate solubilizing microorganisms in sustainable agriculture-a review. *Sustainable Agriculture* **27**(1): 551-570.

Khare E, Arora NK. 2011. Dual activity of pyocyanin from *Pseudomonas aeruginosa*—antibiotic against phytopathogen and signal molecule for biofilm development by rhizobia. *Canadian Journal of Microbiology* **57**(9): 708-713.

Kiers ET, Denison RF. 2008. Sanctions, cooperation, and the stability of plant-rhizosphere mutualisms. *Annual Review of Ecology, Evolution, and Systematics*: 215-236.

Kiers ET, Rousseau RA, West SA, Denison RF. 2003. Host sanctions and the legume–rhizobium mutualism. *Nature* **425**(6953): 78-81.

Knudson L. 1920. The secretion of invertase by plant roots. *American Journal of Botany* **7**(9): 371-379.

Kong Z, Deng Z, Glick BR, Wei G, Chou M. 2017. A nodule endophytic plant growth-promoting *Pseudomonas* and its effects on growth, nodulation and metal uptake in *Medicago lupulina* under copper stress. *Annals of Microbiology* **67**(1): 49-58.

Kraiser T, Gras DE, Gutiérrez AG, González B, Gutiérrez RA. 2011. A holistic view of nitrogen acquisition in plants. *Journal of Experimental Botany* **62**(4): 1455-1466.

Kraut-Cohen J, Zolti A, Shaltiel-Harpaz L, Argaman E, Rabinovich R, Green SJ, Minz D. 2020. Effects of tillage practices on soil microbiome and agricultural parameters. *Science of The Total Environment* **705**: 135791.

Kumawat K, Sharma P, Sirari A, Singh I, Gill B, Singh U, Saharan K. 2019. Synergism of *Pseudomonas aeruginosa* (LSE-2) nodule endophyte with *Bradyrhizobium* sp.(LSBR-3) for improving plant growth, nutrient acquisition and soil health in soybean. *World Journal of Microbiology and Biotechnology* **35**(3): 1-17.

Kusakabe S, Higasitani N, Kaneko T, Yasuda M, Miwa H, Okazaki S, Saeki K, Higashitani A, Sato S. 2020. *Lotus* accessions possess multiple checkpoints triggered by different type III secretion system effectors of the wide-host-range symbiont *Bradyrhizobium elkanii* USDA61. *Microbes and Environments* **35**(1): ME19141.

Lauber CL, Hamady M, Knight R, Fierer N. 2009. Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Applied and Environmental Microbiology* **75**(15): 5111-5120.

Lauber CL, Strickland MS, Bradford MA, Fierer N. 2008. The influence of soil properties on the structure of bacterial and fungal communities across land-use types. *Soil Biology and Biochemistry* **40**(9): 2407-2415.

Leghari SJ, Wahocho NA, Laghari GM, HafeezLaghari A, MustafaBhabhan G, HussainTalpur K, Bhutto TA, Wahocho SA, Lashari AA. 2016. Role of nitrogen for plant growth and development: A review. *Advances in Environmental Biology* **10**(9): 209-219.

Leite J, Fischer D, Rouws LF, Fernandes-Júnior PI, Hofmann A, Kublik S, Schloter M, Xavier GR, Radl V. 2017. Cowpea nodules harbor non-rhizobial bacterial communities that are shaped by soil type rather than plant genotype. *Frontiers in Plant Science* **7**: 2064.

Lerouge P, Roche P, Faucher C, Maillet F, Truchet G, Promé JC, Dénarié J. 1990. Symbiotic host-specificity of *Rhizobium meliloti* is determined by a sulphated and acylated glucosamine oligosaccharide signal. *Nature* **344**(6268): 781-784.

Li D-M, Alexander M. 1986. Bacterial growth rates and competition affect nodulation and root colonization by *Rhizobium meliloti*. *Applied and Environmental Microbiology* **52**(4): 807-811.

Li Y, Li X, Liu Y, Wang ET, Ren C, Liu W, Xu H, Wu H, Jiang N, Li Y. 2016. Genetic diversity and community structure of rhizobia nodulating *Sesbania cannabina* in saline–alkaline soils. *Systematic and Applied Microbiology* **39**(3): 195-202.

Liang J. 2020. *The Rhizobium leguminosarum Norway - Lotus burttii intraction as a model for studying how rhizobia enter plant cells.* PhD, Ludwig-Maximilians University.

Liang J, Hoffrichter A, Brachmann A, Marín M. 2018. Complete genome of *Rhizobium leguminosarum* Norway, an ineffective Lotus micro-symbiont. *Standards in Genomic Sciences* **13**(1): 1-11.

Liang J, Klingl A, Lin Y-Y, Boul E, Thomas-Oates J, Marín M. 2019. A subcompatible rhizobium strain reveals infection duality in Lotus. *Journal of Experimental Botany* **70**(6): 1903-1913.

Ling J, Zheng H, Katzianer DS, Wang H, Zhong Z, Zhu J. 2013. Applying reversible mutations of nodulation and nitrogen-fixation genes to study social cheating in *Rhizobium etli*-legume interaction. *PLoS One* **8**(7): e70138.

Liu F, Hewezi T, Lebeis SL, Pantalone V, Grewal PS, Staton ME. 2019. Soil indigenous microbiome and plant genotypes cooperatively modify soybean rhizosphere microbiome assembly. *BMC Microbiology* **19**(1): 1-19.

Liu F, Xing S, Ma H, Du Z, Ma B. 2013. Cytokinin-producing, plant growth-promoting rhizobacteria that confer resistance to drought stress in *Platycladus orientalis* container seedlings. *Applied Microbiology and Biotechnology* **97**(20): 9155-9164.

Liu J, Yu X, Qin Q, Dinkins RD, Zhu H. 2020. The impacts of domestication and breeding on nitrogen fixation symbiosis in legumes. *Frontiers in Genetics* **11**: 00973.

Long SR. 1996. *Rhizobium* symbiosis: nod factors in perspective. *The Plant Cell* **8**(10): 1885.

Lugtenberg B, Kamilova F. 2009. Plant-growth-promoting rhizobacteria. *Annual Review of Microbiology* **63**(1): 541-556.

Lyu D, Zajonc J, Pagé A, Tanney CA, Shah A, Monjezi N, Msimbira LA, Antar M, Nazari M, Backer R. 2021. Plant holobiont theory: The phytomicrobiome plays a central role in evolution and success. *Microorganisms* **9**(4): 675.

Malik DK, Sindhu SS. 2011. Production of indole acetic acid by *Pseudomonas* sp.: effect of coinoculation with *Mesorhizobium* sp. *Cicer* on nodulation and plant growth of chickpea (*Cicer arietinum*). *Physiology and Molecular Biology of Plants* **17**(1): 25-32.

Mallon CA, Poly F, Le Roux X, Marring I, van Elsas JD, Salles JF. 2015. Resource pulses can alleviate the biodiversity–invasion relationship in soil microbial communities. *Ecology* **96**(4): 915-926.

Markmann K, Radutoiu S, Stougaard J 2012. Infection of *Lotus japonicus* roots by *Mesorhizobium loti*. *Signaling and Communication in Plant Symbiosis*: Springer, 31-50.

Martínez-Hidalgo P, García JM, Pozo MJ. 2015. Induced systemic resistance against *Botrytis cinerea* by *Micromonospora* strains isolated from root nodules. *Frontiers in Microbiology* **6**: 922.

Martínez-Hidalgo P, Hirsch AM. 2017. The nodule microbiome: N2-fixing rhizobia do not live alone. *Phytobiomes Journal* **1**(2): 70-82.

Massalha H, Korenblum E, Malitsky S, Shapiro OH, Aharoni A. 2017. Live imaging of root–bacteria interactions in a microfluidics setup. *Proceedings of the National Academy of Sciences* **114**(17): 4549-4554.

Masson-Boivin C, Sachs JL. 2018. Symbiotic nitrogen fixation by rhizobia—the roots of a success story. *Current Opinion in Plant Biology* **44**: 7-15.

Mayer Z, Sasvári Z, Szentpéteri V, Pethőné Rétháti B, Vajna B, Posta K. 2019. Effect of long-term cropping systems on the diversity of the soil bacterial communities. *Agronomy* **9**(12): 878.

Mayhood P, Mirza BS. 2021. Soybean root nodule and rhizosphere microbiome: Distribution of rhizobial and nonrhizobial endophytes. *Applied and Environmental Microbiology* **87**(10): e02884-02820.

Mendes R, Kruijt M, De Bruijn I, Dekkers E, Van Der Voort M, Schneider JH, Piceno YM, DeSantis TZ, Andersen GL, Bakker PA. 2011. Deciphering the rhizosphere microbiome for disease-suppressive bacteria. *Science* **332**(6033): 1097-1100.

Mergaert P, Van Montagu M, Holsters M. 1997. Molecular mechanisms of Nod factor diversity. *Molecular Microbiology* **25**(5): 811-817.

Mezaache-Aichour S, Guechi A, Zerroug M, Nicklin J, Strange R. 2013. Antimicrobial activity of *Pseudomonas* secondary metabolites. *Pharmacognosy Communications* **3**(3).

Montiel J, Reid D, Grønbæk TH, Benfeldt CM, James EK, Ott T, Ditengou FA, Nadzieja M, Kelly S, Stougaard J. 2021. Distinct signaling routes mediate intercellular and intracellular rhizobial infection in *Lotus japonicus*. *Plant Physiology* **185**(3): 1131-1147.

Moyano G, Marco D, Knopoff D, Torres G, Turner C. 2017. Explaining coexistence of nitrogen fixing and non-fixing rhizobia in legume-rhizobia mutualism using mathematical modeling. *Mathematical Biosciences* **292**: 30-35.

Mukhtar S, Hirsch AM, Khan N, Malik KA, Humm EA, Pellegrini M, Shi B, Briscoe L, Huntemann M, Clum A. 2020. Impact of soil salinity on the cowpea nodule-microbiome and the isolation of halotolerant PGPR strains to promote plant growth under salinity stress. *Phytobiomes Journal* **4**(4): 364-374.

Müller M, Munné-Bosch S. 2015. Ethylene response factors: a key regulatory hub in hormone and stress signaling. *Plant Physiology* **169**(1): 32-41.

Munoz-Ucros J, Zwetsloot MJ, Cuellar-Gempeler C, Bauerle TL. 2021. Spatiotemporal patterns of rhizosphere microbiome assembly: From ecological theory to agricultural application. *Journal of Applied Ecology* **58**(5): 894-904.

Muresu R, Porceddu A, Sulas L, Squartini A. 2019. Nodule-associated microbiome diversity in wild populations of *Sulla coronaria* reveals clues on the relative importance of culturable rhizobial symbionts and co-infecting endophytes. *Microbiological Research* **221**: 10-14.

Mus F, Crook MB, Garcia K, Garcia Costas A, Geddes BA, Kouri ED, Paramasivan P, Ryu M-H, Oldroyd GE, Poole PS. 2016. Symbiotic nitrogen fixation and the challenges to its extension to nonlegumes. *Applied and Environmental Microbiology* **82**(13): 3698-3710.

Mutch LA, Young JPW. 2004. Diversity and specificity of *Rhizobium leguminosarum* biovar *viciae* on wild and cultivated legumes. *Molecular Ecology* **13**(8): 2435-2444.

Nandasena KG, O'Hara GW, Tiwari RP, Sezmiş E, Howieson JG. 2007. *In situ* lateral transfer of symbiosis islands results in rapid evolution of diverse competitive strains of mesorhizobia suboptimal in symbiotic nitrogen fixation on the pasture legume *Biserrula pelecinus* L. *Environmental Microbiology* **9**(10): 2496-2511.

Nandasena KG, O'Hara GW, Tiwari RP, Willems A, Howieson JG. 2009. *Mesorhizobium australicum* sp. nov. and *Mesorhizobium opportunistum* sp. nov., isolated from *Biserrula pelecinus* L. in Australia. *International Journal of Systematic and Evolutionary Microbiology* **59**(9): 2140-2147.

Nascimento F, Brígido C, Glick B, Oliveira S, Alho L. 2012. *Mesorhizobium ciceri* LMS-1 expressing an exogenous 1-aminocyclopropane-1-carboxylate (ACC) deaminase increases its nodulation abilities and chickpea plant resistance to soil constraints. *Letters in Applied Microbiology* **55**(1): 15-21.

Ndlovu J, Richardson DM, Wilson JR, Le Roux JJ. 2013. Co-invasion of South African ecosystems by an Australian legume and its rhizobial symbionts. *Journal of Biogeography* **40**(7): 1240-1251.

Nett RS, Bender KS, Peters RJ. 2022. Production of the plant hormone gibberellin by rhizobia increases host legume nodule size. *The ISME Journal*: 1-9.

Noble WS. 2006. What is a support vector machine? *Nature biotechnology* **24**(12): 1565-1567.

Ofori F, Stern W. 1987. Cereal–legume intercropping systems. *Advances in Agronomy* **41**: 41-90.

Oldroyd GE, Engstrom EM, Long SR. 2001. Ethylene inhibits the Nod factor signal transduction pathway of *Medicago truncatula*. *The Plant Cell* **13**(8): 1835-1849.

Ott T, van Dongen JT, Gu C, Krusell L, Desbrosses G, Vigeolas H, Bock V, Czechowski T, Geigenberger P, Udvardi MK. 2005. Symbiotic leghemoglobins are crucial for nitrogen fixation in legume root nodules but not for general plant growth and development. *Current Biology* **15**(6): 531-535.

Pandya M, Naresh Kumar G, Rajkumar S. 2013. Invasion of rhizobial infection thread by non-rhizobia for colonization of *Vigna radiata* root nodules. *FEMS Microbiology Letters* **348**(1): 58-65.

Papanastasis V, Arianoutsou M, Lyrintzis G 2004. Management of biotic resources in ancient Greece. *Proceedings of the 10th Mediterranean Ecosystems (MEDECOS) Conference*. 1-11.

Parniske M, Schmidt P, Kosch K, Müller P. 1994. Plant defense responses of host plants with determinate nodules induced by eps-defective exob mutants of *Bradyrhizobium japonicum*. *Molecular Plant-Microbe Interactions*(5): 631-638.

Pastor-Bueis R, Jimenez-Gomez A, Barquero M, Mateos PF, González-Andrés F. 2021. Yield response of common bean to co-inoculation with *Rhizobium* and *Pseudomonas*

endophytes and microscopic evidence of different colonised spaces inside the nodule. *European Journal of Agronomy* **122**: 126187.

Pawlik M, Cania B, Thijs S, Vangronsveld J, Piotrowska-Seget Z. 2017. Hydrocarbon degradation potential and plant growth-promoting activity of culturable endophytic bacteria of *Lotus corniculatus* and *Oenothera biennis* from a long-term polluted site. *Environmental Science and Pollution Research* **24**(24): 19640-19652.

Peoples M, Brockwell J, Herridge D, Rochester I, Alves B, Urquiaga S, Boddey R, Dakora F, Bhattarai S, Maskey S. 2009. The contributions of nitrogen-fixing crop legumes to the productivity of agricultural systems. *Symbiosis* **48**(1): 1-17.

Perret X, Staehelin C, Broughton WJ. 2000. Molecular basis of symbiotic promiscuity. *Microbiology and Molecular Biology Reviews* **64**(1): 180-201.

Pester M, Bittner N, Deevong P, Wagner M, Loy A. 2010. A 'rare biosphere'microorganism contributes to sulfate reduction in a peatland. *The ISME Journal* **4**(12): 1591-1602.

Preinsberger A. 2021. *Characterization of Pseudomonas strains isolated from root nodules of healthy Lotus burttii plants.* Bachelor, Ludwig-Maximilians University.

Pule-Meulenberg F, Belane AK, Krasova-Wade T, Dakora FD. 2010. Symbiotic functioning and bradyrhizobial biodiversity of cowpea (*Vigna unguiculata* L. Walp.) in Africa. *BMC Microbiology* **10**(1): 1-12.

Quides KW, Stomackin GM, Lee H-H, Chang JH, Sachs JL. 2017. *Lotus japonicus* alters in planta fitness of *Mesorhizobium loti* dependent on symbiotic nitrogen fixation. *PLoS One* **12**(9): e0185568.

Ramey BE, Koutsoudis M, von Bodman SB, Fuqua C. 2004. Biofilm formation in plant–microbe associations. *Current Opinion in Microbiology* **7**(6): 602-609.

Reeve W, Nandasena K, Yates R, Tiwari R, O'Hara G, Ninawi M, Chertkov O, Goodwin L, Bruce D, Detter C. 2013. Complete genome sequence of *Mesorhizobium opportunistum* type strain WSM2075T. *Standards in Genomic Sciences* **9**(2): 294-303.

Regus JU, Quides KW, O'Neill MR, Suzuki R, Savory EA, Chang JH, Sachs JL. 2017. Cell autonomous sanctions in legumes target ineffective rhizobia in nodules with mixed infections. *American Journal of Botany* **104**(9): 1299-1312.

Rocha SMB, Mendes LW, de Souza Oliveira LM, Melo VMM, Antunes JEL, Araujo FF, Hungria M, Araujo ASF. 2020. Nodule microbiome from cowpea and lima bean grown in composted tannery sludge-treated soil. *Applied Soil Ecology* **151**: 103542.

Rodpothong P, Sullivan JT, Songsrirote K, Sumpton D, Cheung KW-T, Thomas-Oates J, Radutoiu S, Stougaard J, Ronson CW. 2009. Nodulation gene mutants of *Mesorhizobium loti* R7A—*nodZ* and *nolL* mutants have host-specific phenotypes on *Lotus* spp. *Molecular Plant-Microbe Interactions* **22**(12): 1546-1554.

Rolli E, Marasco R, Vigani G, Ettoumi B, Mapelli F, Deangelis ML, Gandolfi C, Casati E, Previtali F, Gerbino R. 2015. Improved plant resistance to drought is promoted by the root-associated microbiome as a water stress-dependent trait. *Environmental Microbiology* **17**(2): 316-331.

Ryu M-H, Zhang J, Toth T, Khokhani D, Geddes BA, Mus F, Garcia-Costas A, Peters JW, Poole PS, Ané J-M. 2020. Control of nitrogen fixation in bacteria that associate with cereals. *Nature Microbiology* **5**(2): 314-330.

Sachs JL, Simms EL. 2006. Pathways to mutualism breakdown. *Trends in Ecology & Evolution* **21**(10): 585-592.

Sachs JL, Simms EL. 2008. The origins of uncooperative rhizobia. *Oikos* **117**(7): 961-966.

Saleem M, Law AD, Sahib MR, Pervaiz ZH, Zhang Q. 2018. Impact of root system architecture on rhizosphere and root microbiome. *Rhizosphere* **6**: 47-51.

Sánchez-Cañizares C, Jorrín B, Poole PS, Tkacz A. 2017. Understanding the holobiont: the interdependence of plants and their microbiome. *Current Opinion in Microbiology* **38**: 188-196.

Sánchez AC, Gutiérrez RT, Santana RC, Urrutia AR, Fauvart M, Michiels J, Vanderleyden J. 2014. Effects of co-inoculation of native *Rhizobium* and *Pseudomonas* strains on growth parameters and yield of two contrasting *Phaseolus vulgaris* L. genotypes under Cuban soil conditions. *European Journal of Soil Biology* **62**: 105-112.

Santos MS, Nogueira MA, Hungria M. 2019. Microbial inoculants: reviewing the past, discussing the present and previewing an outstanding future for the use of beneficial bacteria in agriculture. *Amb Express* **9**(1): 1-22.

Sasse J, Martinoia E, Northen T. 2018. Feed your friends: do plant exudates shape the root microbiome? *Trends in Plant Science* **23**(1): 25-41.

Sayyed R, Chincholkar S, Reddy M, Gangurde N, Patel P 2013. Siderophore producing PGPR for crop nutrition and phytopathogen suppression. *Bacteria in Agrobiology: Disease Management*: Springer, 449-471.

Schlicht M, Ludwig-Müller J, Burbach C, Volkmann D, Baluska F. 2013. Indole-3-butyric acid induces lateral root formation via peroxisome-derived indole-3-acetic acid and nitric oxide. *New Phytologist* **200**(2): 473-482.

Schumpp O, Crevecoeur M, Broughton WJ, Deakin WJ. 2009. Delayed maturation of nodules reduces symbiotic effectiveness of the *Lotus japonicus–Rhizobium* sp. NGR234 interaction. *Journal of Experimental Botany* **60**(2): 581-590.

Schumpp O, Deakin WJ. 2010. How inefficient rhizobia prolong their existence within nodules. *Trends in Plant Science* **15**(4): 189-195.

Scott JE, Li K, Filkins LM, Zhu B, Kuchma SL, Schwartzman JD, O'Toole GA. 2019. *Pseudomonas aeruginosa* can inhibit growth of streptococcal species via siderophore production. *Journal of Bacteriology* **201**(8): e00014-00019.

Selvakumar G, Kundu S, Gupta AD, Shouche YS, Gupta HS. 2008. Isolation and characterization of nonrhizobial plant growth promoting bacteria from nodules of Kudzu (*Pueraria thunbergiana*) and their effect on wheat seedling growth. *Current Microbiology* **56**(2): 134-139.

Serova TA, Tsyganova AV, Tsyganov VE. 2018. Early nodule senescence is activated in symbiotic mutants of pea (*Pisum sativum* L.) forming ineffective nodules blocked at different nodule developmental stages. *Protoplasma* **255**(5): 1443-1459.

Shade A, Jones SE, Caporaso JG, Handelsman J, Knight R, Fierer N, Gilbert JA. 2014. Conditionally rare taxa disproportionately contribute to temporal changes in microbial diversity. *MBio* **5**(4): e01371-01314.

Shah A, Wakelin S, Moot DJ, Blond C, Laugraud A, Ridgway HJ. 2021. *Trifolium repens* and *T. subterraneum* modify their nodule microbiome in response to soil pH. *Journal of Applied Microbiology* **131**(4): 1858-1869.

Sharaf H, Rodrigues RR, Moon J, Zhang B, Mills K, Williams MA. 2019. Unprecedented bacterial community richness in soybean nodules vary with cultivar and water status. *Microbiome* **7**(1): 1-18.

Sharma V, Bhattacharyya S, Kumar R, Kumar A, Ibañez F, Wang J, Guo B, Sudini HK, Gopalakrishnan S, DasGupta M. 2020. Molecular basis of root nodule symbiosis

between *Bradyrhizobium* and 'crack-entry'legume groundnut (*Arachis hypogaea* L.). *Plants* **9**(2): 276.

Shin W, Islam R, Benson A, Joe MM, Kim K, Gopal S, Samaddar S, Banerjee S, Sa T. 2016. Role of diazotrophic bacteria in biological nitrogen fixation and plant growth improvement. *Korean Journal of Soil Science and Fertilizer* **49**(1): 17-29.

Shobha G, Kumudini B. 2012. Antagonistic effect of the newly isolated PGPR *Bacillus* spp. on *Fusarium oxysporum*. *International Journal Applied Science & Engineering Research* **1**(3): 463-474.

Simms EL, Taylor DL. 2002. Partner choice in nitrogen-fixation mutualisms of legumes and rhizobia. *Integrative and Comparative Biology* **42**(2): 369-380.

Simsek S, Ojanen-Reuhs T, Stephens SB, Reuhs BL. 2007. Strain-ecotype specificity in *Sinorhizobium meliloti-Medicago truncatula* symbiosis is correlated to succinoglycan oligosaccharide structure. *Journal of Bacteriology* **189**(21): 7733-7740.

Sindhu S, Dadarwal K. 2001. Chitinolytic and cellulolytic *Pseudomonas* sp. antagonistic to fungal pathogens enhances nodulation by *Mesorhizobium* sp. *Cicer* in chickpea. *Microbiological Research* **156**(4): 353-358.

Singh S, Varma A 2017. Structure, function, and estimation of leghemoglobin. *Rhizobium Biology and Biotechnology*: Springer, 309-330.

Soares R, Trejo J, Lorite MJ, Figueira E, Sanjuán J, Videira e Castro I. 2020. Diversity, phylogeny and plant growth promotion traits of nodule associated bacteria isolated from *Lotus parviflorus*. *Microorganisms* **8**(4): 499.

Sokol NW, Slessarev E, Marschmann GL, Nicolas A, Blazewicz SJ, Brodie EL, Firestone MK, Foley MM, Hestrin R, Hungate BA. 2022. Life and death in the soil microbiome: How ecological processes influence biogeochemistry. *Nature Reviews Microbiology* **20**(1): 415-430.

Sprent JI. 2007. Evolving ideas of legume evolution and diversity: a taxonomic perspective on the occurrence of nodulation. *The New Phytologist* **174**(1): 11-25.

Sprent JI. 2008. 60Ma of legume nodulation. What's new? What's changing? *Journal of Experimental Botany* **59**(5): 1081-1084.

Subba-Rao N, Mateos PF, Baker D, Stuart Pankratz H, Palma J, Dazzo FB, Sprent JI. 1995. The unique root-nodule symbiosis between *Rhizobium* and the aquatic legume, *Neptunia natans* (L. f.) Druce. *Planta* **196**(2): 311-320.

Tapia-García EY, Hernández-Trejo V, Guevara-Luna J, Rojas-Rojas FU, Arroyo-Herrera I, Meza-Radilla G, Vásquez-Murrieta MS, Estrada-de Los Santos P. 2020. Plant growth-promoting bacteria isolated from wild legume nodules and nodules of *Phaseolus vulgaris* L. trap plants in central and southern Mexico. *Microbiological Research* **239**: 126522.

Tariq M, Hameed S, Yasmeen T, Zahid M, Zafar M. 2014. Molecular characterization and identification of plant growth promoting endophytic bacteria isolated from the root nodules of pea (*Pisum sativum* L.). *World Journal of Microbiology and Biotechnology* **30**(2): 719-725.

Thakur MP, Geisen S. 2019. Trophic regulations of the soil microbiome. *Trends in Microbiology* **27**(9): 771-780.

Tkacz A, Cheema J, Chandra G, Grant A, Poole PS. 2015. Stability and succession of the rhizosphere microbiota depends upon plant type and soil composition. *The ISME Journal* **9**(11): 2349-2359.

Trivedi P, Leach JE, Tringe SG, Sa T, Singh BK. 2020. Plant–microbiome interactions: from community assembly to plant health. *Nature Reviews Microbiology* **18**(11): 607-621.

Van Der Heijden MG, Bruin Sd, Luckerhoff L, Van Logtestijn RS, Schlaeppi K. 2016. A widespread plant-fungal-bacterial symbiosis promotes plant biodiversity, plant nutrition and seedling recruitment. *The ISME Journal* **10**(2): 389-399.

van Rhijn P, Vanderleyden J. 1995. The *Rhizobium*-plant symbiosis. *Microbiological Reviews* **59**(1): 124-142.

Vandenkoornhuyse P, Quaiser A, Duhamel M, Le Van A, Dufresne A. 2015. The importance of the microbiome of the plant holobiont. *New Phytologist* **206**(4): 1196-1206.

Velázquez E, Carro L, Flores-Félix JD, Martínez-Hidalgo P, Menéndez E, Ramírez-Bahena M-H, Mulas R, González-Andrés F, Martínez-Molina E, Peix A 2017. The legume nodule microbiome: a source of plant growth-promoting bacteria. *Probiotics and Plant Health*: Springer, 41-70.

Venado RE, Liang J, Marín M 2020. Rhizobia infection, a journey to the inside of plant cells. *Advances in Botanical Research*: Elsevier, 97-118.

Verma J, Yadav J, Tiwari K, Lavakush S, Singh V. 2010. Impact of plant growth promoting rhizobacteria on crop production. *International Journal of Agricultural Research* **5**(11): 954-983.

Viands D, Vance C, Heichel G, Barnes D. 1979. An Ineffective Nitrogen Fixation Trait in Alfalfa 1. *Crop Science* **19**(6): 905-908.

Wang D, Yang S, Tang F, Zhu H. 2012. Symbiosis specificity in the legume–rhizobial mutualism. *Cellular Microbiology* **14**(3): 334-342.

Wang LL, Wang ET, Liu J, Li Y, Chen WX. 2006. Endophytic occupation of root nodules and roots of *Melilotus dentatus* by *Agrobacterium tumefaciens*. *Microbial Ecology* **52**(3): 436-443.

Wang S, Fan H, Zhao D, Zhu X, Wang Y, Liu X, Liu D, Duan Y, Chen L. 2021. Multifunctional efficacy of the nodule endophyte *Pseudomonas fragi* in stimulating tomato immune response against *Meloidogyne incognita*. *Biological Control* **164**: 104773.

Wang W, Hu M, Shu X, Li H, Qi W, Yang Y, Zhang Q. 2021. Microbiome of permeable sandy substrate in headwater river is shaped by water chemistry rather than grain size and heterogeneity. *Science of The Total Environment* **780**: 146552.

Westhoek A, Field E, Rehling F, Mulley G, Webb I, Poole PS, Turnbull LA. 2017. Policing the legume-*Rhizobium* symbiosis: a critical test of partner choice. *Scientific Reports* **7**(1): 1-10.

Wiedenbeck J, Cohan FM. 2011. Origins of bacterial diversity through horizontal genetic transfer and adaptation to new ecological niches. *FEMS Microbiology Reviews* **35**(5): 957-976.

Windels EM, Van den Bergh B, Michiels J. 2020. Bacteria under antibiotic attack: different strategies for evolutionary adaptation. *PLoS Pathogens* **16**(5): e1008431.

Wu Y, Zeng J, Zhu Q, Zhang Z, Lin X. 2017. pH is the primary determinant of the bacterial community structure in agricultural soils impacted by polycyclic aromatic hydrocarbon pollution. *Scientific Reports* **7**(1): 1-7.

Xiong Y-W, Li X-W, Wang T-T, Gong Y, Zhang C-M, Xing K, Qin S. 2020. Root exudates-driven rhizosphere recruitment of the plant growth-promoting rhizobacterium *Bacillus flexus* KLBMP 4941 and its growth-promoting effect on the coastal halophyte *Limonium sinense* under salt stress. *Ecotoxicology and Environmental Safety* **194**: 110374.

Yadegari M, Rahmani HA. 2010. Evaluation of bean(*Phaseolus vulgaris*) seeds' inoculation with *Rhizobium phaseoli* and plant growth promoting rhizobacteria(PGPR) on yield and yield components. *African Journal of Agricultural Research* **5**(9): 792-799.

Zahran HH. 2001. Rhizobia from wild legumes: diversity, taxonomy, ecology, nitrogen fixation and biotechnology. *Journal of Biotechnology* **91**(2-3): 143-153.

Zarrabian M, Montiel J, Sandal N, Jin H, Lin Y-Y, Klingl V, Marín M, James E, Parniske M, Stougaard J. 2021. A promiscuity locus confers *Lotus burttii* nodulation with rhizobia from five different genera. *bioRxiv*.

Zarraonaindia I, Owens SM, Weisenhorn P, West K, Hampton-Marcell J, Lax S, Bokulich NA, Mills DA, Martin G, Taghavi S. 2015. The soil microbiome influences grapevine-associated microbiota. *Mbio* **6**(2): e02527-02514.

Zgadzaj R, Garrido-Oter R, Jensen DB, Koprivova A, Schulze-Lefert P, Radutoiu S. 2016. Root nodule symbiosis in *Lotus japonicus* drives the establishment of distinctive rhizosphere, root, and nodule bacterial communities. *Proceedings of the National Academy of Sciences* **113**(49): E7996-E8005.

Zgadzaj R, James EK, Kelly S, Kawaharada Y, de Jonge N, Jensen DB, Madsen LH, Radutoiu S. 2015. A legume genetic framework controls infection of nodules by symbiotic and endophytic bacteria. *PLoS Genetics* **11**(6): e1005280.

Zhalnina K, Dias R, de Quadros PD, Davis-Richardson A, Camargo FA, Clark IM, McGrath SP, Hirsch PR, Triplett EW. 2015. Soil pH determines microbial diversity and composition in the park grass experiment. *Microbial Ecology* **69**(2): 395-406.

Zhalnina K, Louie KB, Hao Z, Mansoori N, da Rocha UN, Shi S, Cho H, Karaoz U, Loqué D, Bowen BP. 2018. Dynamic root exudate chemistry and microbial substrate preferences drive patterns in rhizosphere microbial community assembly. *Nature Microbiology* **3**(4): 470-480.

Zhang JJ, Yu T, Lou K, Mao PH, Wang ET, Chen WF, Chen WX. 2014. Genotypic alteration and competitive nodulation of *Mesorhizobium muleiense* against exotic chickpea rhizobia in alkaline soils. *Systematic and Applied Microbiology* **37**(7): 520-524.

Zhang YM, Li Jr Y, Chen WF, Wang ET, Tian CF, Li QQ, Zhang YZ, Sui XH, Chen WX. 2011. Biodiversity and biogeography of rhizobia associated with soybean plants grown in the North China Plain. *Applied and Environmental Microbiology* **77**(18): 6331-6342.

Zhao LF, Xu YJ, Ma ZQ, Deng ZS, Shan CJ, Wei GH. 2013. Colonization and plant growth promoting characterization of endophytic *Pseudomonas chlororaphis* strain Zong1 isolated from *Sophora alopecuroides* root nodules. *Brazilian Journal of Microbiology* **44**: 629-637.

Zheng Y, Liang J, Zhao D-L, Meng C, Xu Z-C, Xie Z-H, Zhang C-S. 2020. The root nodule microbiome of cultivated and wild halophytic legumes showed similar diversity but distinct community structure in Yellow River Delta saline soils. *Microorganisms* **8**(2): 207.

Zhu D, Ding J, Yin Y, Ke X, O'Connor P, Zhu Y-G. 2020. Effects of earthworms on the microbiomes and antibiotic resistomes of detritus fauna and phyllospheres. *Environmental Science & Technology* **54**(10): 6000-6008.

Zilber-Rosenberg I, Rosenberg E. 2008. Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. *FEMS Microbiology Reviews* **32**(5): 723-735.

Acknowledgements

I would first like to thank my supervisor Dr. Macarena Marín. Initially for warmly welcoming me into the group to carry out my Master thesis and then offering me the opportunity to continue on as a doctoral candidate. The support she provided with the technical side of the lab work has been invaluable and has improved my skills dramatically. I would also like to thank her for always being available for a stimulating discussion in reinvigorate my motivation for this project as well as being receptive to a moment of levity to keep the mood friendly.

Secondly, I would like to thank all our collaborators who assisted in the project. Maryam Mahmoudi and Prof. Dr. Eric Kemen for their advice and analysis of computational data. Dr. Vivian Radl and Prof. Dr. Andreas Brachmann for their assistance in sequencing. Also Prof. Dr. Michael Schloter for his assistance in constructing the final manuscript of our publication. I would also like to thank Tolganay Kabdullayeva for leading the first publication.

I would also like to thank the members of my thesis advisory committee, Prof. Dr. Heinrich Jung and Prof. Dr. Claude Becker, for their ongoing support and constructive feedback throughout the committee meetings. I would also like to thank Prof. Dr. Andreas Klingl, Prof. Dr. Wolfgang Frank and Prof. Dr. Richard Merrill for marking this thesis.

I want to thank all the members of AG Marín over the past few years. Especially Rafael Venado, Juan Liang and Yen-Yu Lin for their help in the lab and their willingness to assist me when they were able. I would also like to thank the entire genetics department of LMU for the friendliness and cooperation.

I want to thank my family, especially my father, who, although far away, were always available for encouragement and advice. I would also to thank all my friends outside the lab who, at times, provided a silly and welcoming distraction from my project as well as facilitating my love of Munich.

Lastly, I would like to thank my wife Isabel for her love and support over the years. This project has been wonderful, but landing you has been my greatest achievement.