Host regulation of the legume-rhizobia symbiosis



Laura Jane Clark
Merton College
University of Oxford

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0.1 Declaration of Authorship

I declare that all parts of this thesis are my own work, except for some data in **Chapter 3** which are the work of contributing researchers: Andrzej Tkacz, Annet Westhoek, and Megan Griffiths; all University of Oxford. Details of these contributions are given in **Chapter 3**.

All photographs were taken by John Baker, University of Oxford.

This thesis has not been submitted, either partially or in full, for any degree at this University or another institution.

Laura J Clark

0.2 Acknowledgements

Despite having only one name on the front cover, this thesis would not have been possible without the contributions and support of many incredible people.

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0.3 Abstract

Leguminous plants host nitrogen-fixing bacteria, known as rhizobia, in specialised root nodules, providing them with carbon in return for nitrogen. However, plants face an investment dilemma, as they usually house multiple strains of rhizobia that can vary in their fixation ability. This thesis characterises the host plant response to poorly fixing strains and compares this across nodule types.

While plants are known to sanction non-fixing strains, non-fixers are rare in field settings, while intermediate fixers are common. We showed that pea (*Pisum sativum*) plants tolerated an otherwise isogenic intermediate fixer only when a better strain was unavailable. Nodules containing the intermediate fixer were large and healthy when the only alternative was a non-fixer. When a more effective strain was available, carbon transport to the intermediate fixer was decreased, nodules were smaller, with reduced nitrogen fixation, and contained fewer viable rhizobia. Flow cytometry showed that sanctioning hinders nodule development, as sanctioned nodules have fewer specialised nitrogen-fixing bacteroids. Furthermore, limited evidence of sanctioning was observed within mixed nodules, containing two strains, suggesting that poorly fixing strains may not be able to evade sanctioning by sharing a nodule with a better strain. This indicates that legumes can make conditional decisions at a per-nodule level, and possibly even within nodules, giving them remarkable control over their symbiotic partners.

We compared hosts with indeterminate (pea, *Pisum sativum*) and determinate (bean, *Phaseolus vulgaris*) nodules, to show that for strains differing only in nitrogen-fixation, the mechanism of host regulation was sanctioning (reducing resource allocation), rather than partner choice (forming fewer nodules). This opens avenues for further work investigating sanctioning mechanisms in both indeterminate and determinate nodules. Furthermore, indeterminate fixing nodules were largest when rare, showing an even finer level of host regulation.

These findings improve the understanding of how host plants manage their interactions with multiple symbiotic partners, which is of great importance when developing more effective rhizobia as alternatives to nitrogenous fertilisers.

0.4 Abbreviations

ALA	Aminolevulinic acid
Amp	Ampicillin
Amp^r	Ampicillin resistant
BFP	Blue fluorescent protein
BNF	Biological nitrogen fixation
bp	Base pair
bv.	Biovar
Cb	Carbenicillin
Cb^r	Carbenicillin resistant
CFP	Cyan fluorescent protein
CFU	Colony forming unit
CI	Confidence interval
cps	Counts per second
cv.	Cultivar
DNA	Deoxyribonucleic acid
dpi	Days post inoculation
DWA	Distilled water agar
eCFP	Enhanced cyan fluorescent protein
EDTA	Ethylenediaminetetraacetic acid
eYFP	Enhanced yellow fluorescent protein
gDNA	Genomic deoxyribonucleic acid
Fix^+	Fixing
Fix^{int}	Intermediate fixing
$Fix^–$	Non-fixing
FSC	Forward scatter
GFP	Green fluorescent protein
Gm	Gentamicin
Gm^r	Gentamicin resistant
kb	Kilobases
Km	Kanamycin
Km^r	Kanamycin resistant
LB	Luria Berani broth
Magenta-glcA	5-bromo-6-chloro-3-indolyl-β-D-glucuronide
NCR	Nodule-specific cysteine-rich [peptides]
Nm	Neomycin
Nm^r	Neomycin resistant
Nt	Nitrofurantoin
Nt^r	Nitrofurantoin resistant
ns	Non-significant
Nys	Nystatin
OD	Optical density

OR	Odds ratio
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Rf	Rifampicin
Rf^r	Rifampicin resistant
Rlp	*Rhizobium leguminosarum* bv. *phaseoli*
Rlv	*Rhizobium leguminosarum* bv. *viciae*
RR	Relative risk
rRNA	Ribosomal ribonucleic acid
rpm	Revolutions per minute
SE	Standard error
sfGFP	Superfolder green fluorescent protein
Sp	Spectinomycin
Sp^r	Spectinomycin resistant
SSC	Side scatter
St	Streptomycin
St^r	Streptomycin resistant
Sym	Symbiosis [plasmid]
Tc	Tetracycline
Tc^r	Tetracycline resistant
TY	Tryptone yeast
UV	Ultraviolet
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-glcA	5-bromo-4-chloro-3-indolyl-β-D-glucuronide
YFP	Yellow fluorescent protein

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Chapter 1 Introduction

1.1 Plant-microbe interactions

Plants interact with a variety of microorganisms in the region of soil which surrounds plant roots, known as the rhizosphere[1]. The exudation of photosynthate from plant roots into the rhizosphere facilitates the development of microbial communities[2,3]. Some of these microorganisms can be detrimental to the plant, such as pathogenic fungi, oomycetes[4] and bacteria[5]. Conversely, many of these microorganisms can be beneficial for the plant, from free-living plant-growth-promoting bacteria[6] to the 400 million-year-old mutualism between plants and arbuscular mycorrhizal fungi[7]. These can increase nutrient acquisition[6], stress tolerance[8], or prime plant defences[9], amongst other positive effects[10,11].

Nutrient acquisition is key for plant growth, particularly nitrogen, which is needed for the synthesis of nucleotides, amino acids, proteins, and many secondary compounds[12]. Despite the atmosphere consisting of around 78% N_2 gas, N_2 is highly inert, and therefore most ecosystems are nitrogen limited[13]. The symbiosis between leguminous plants and nitrogen-fixing rhizobia has overcome this limitation. The rhizobia reside in root nodules formed by their host plant, fixing atmospheric dinitrogen into ammonia, which is provided to the host plant in exchange for carbon and other nutrients[14].

Legumes are major food crops, thanks to their symbiosis with rhizobia, as they are high in protein, with the most commonly consumed legumes including common bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), and lentil (*Lens esculenta*)[15]. Legumes are also important for their use in the production of vegetable oils, such as soybean (*Glycine max*) and peanut (*Arachis hypogeae*); and for their use as animal fodder[15]. Additionally, legumes can be planted in rotation with, or alongside, other crops. This can increase soil

nitrogen content and disrupt the cycles of agricultural pests and diseases, contributing to increased crop yields[16].

1.2 Nitrogen

Access to nitrogen is of particular importance to agriculture. The global human population is increasing, estimated to reach around 10 billion people by the year 2050[17]. In order to provide adequate nutrition for this population, agricultural productivity will need to increase by around 70%[18]. This increase in productivity will require the provision of sufficient nutrients for crop plants, including nitrogen[19]. These nutrients can be supplied to crops by the application of artificial fertilisers.

However, heavy application of artificial fertilisers is not a sustainable choice for agriculture. Nitrogenous fertilisers are generated by the Haber-Bosch process, which is energy intensive and responsible for 1.4% of global energy demand[20]. Additionally, the application of high concentrations of fertilisers to agricultural crops is environmentally damaging. Fertilisers can leach into waterways leading to eutrophication and disruption of aquatic ecosystems, and the production of the greenhouse gas, nitrous oxide[21]. Furthermore, artificial fertilisers are often underused in developing nations due to their high cost[22]. Therefore, increasing agricultural productivity with the use of artificial fertilisers alone is difficult and would come with many environmental costs.

Biological nitrogen fixation (BNF) provides a sustainable alternative to the application of artificial nitrogenous fertilises. BNF already contributes greatly to the nitrogen requirements of crops, with the legume-rhizobia symbiosis fixing 40 million tonnes of nitrogen per year[14]. Recent efforts to maximise BNF for agriculture include improving

the efficiency of legume-rhizobia interactions[23], integrating the symbiosis into non-legume crops[24], and developing free-living nitrogen-fixing bacteria which remain in the rhizosphere[25]. Additionally, commercial inoculants of rhizobia are often applied to crops to increase BNF and therefore productivity, particularly in areas with little to no compatible native soil rhizobia, such as Australia[23]. However, these inoculants must compete against or cooperate with native rhizobia and other soil organisms[26], and so sometimes do not persist in the soil[27]. This is problematic as in the field, many native rhizobial strains may fix no or little nitrogen[27–29]. Given the range of native rhizobia which are present in agricultural settings, we must better understand the process of host plant selection and regulation of their bacterial partners in order to develop better inoculants with higher success in the field.

1.3 The legume-rhizobia symbiosis

1.3.1 Legume-rhizobia taxonomy

Nitrogen fixation within root nodules is restricted to four plant orders: the Fabales (which contains the *Fabaceae* or legumes), Fagales, Rosales and Cucurbitales[30]. These orders form the nitrogen-fixing clade, last sharing a common ancestor 100 Mya[30]. The Fabales and one Rosales genus *Parasponia* form associations with rhizobia (α- and β-proteobacteria)[30,31], and the Fagales, Cucurbitales and remaining Rosales form associations with *Frankia*[32].

However, not all plants in the nitrogen-fixing clade can nodulate, and there is a large diversity of infection pathways, nodule structures and associated bacterial species throughout the clade[33]. This gave rise to the generally accepted theory that a precursor adaptation occurred in the last common ancestor of the nitrogen-fixing clade, followed

by several independent gains and losses of the symbiosis[30,33–35]. However two recent comparative genetic studies both suggest a single origin of nodulation, followed by multiple subsequent losses in different lineages, based on the patterns of losses and gains of several key nitrogen-fixing genes[36,37]. Multiple losses of the symbiosis suggest that this interaction is not beneficial for the host plant under certain circumstances (see **1.4.1**).

1.3.2 Nodule formation

Plants acquire their symbiotic partners from the soil after germination, and therefore the potential partners with which a plant can interact is locale-specific[38]. This multi-stage process relies on many signals between plant and rhizobia, as reviewed by Ferguson *et al.* (2010)[39], Oldroyd *et al.* (2011)[40], Oldroyd (2013)[41], and Poole *et al.* (2018)[1] (**Figure 1.1**).

Low soil nitrogen stimulates root exudate production, particularly flavonoids[42], which act as chemoattractant to guide bacterial partners to the root[43,44]. Rhizobia perceive flavonoids via NodD, a transcriptional activator which activates downstream *nod* genes[45], producing lipochitooligosaccharides known as Nod factors[46]. The plant perceives the Nod factors via receptor-like kinases which have a LysM domain[47]. The specific combination of flavonoids and Nod factors, and their perception by rhizobia and plants determines the host range and specificity of rhizobia[43,48,49].

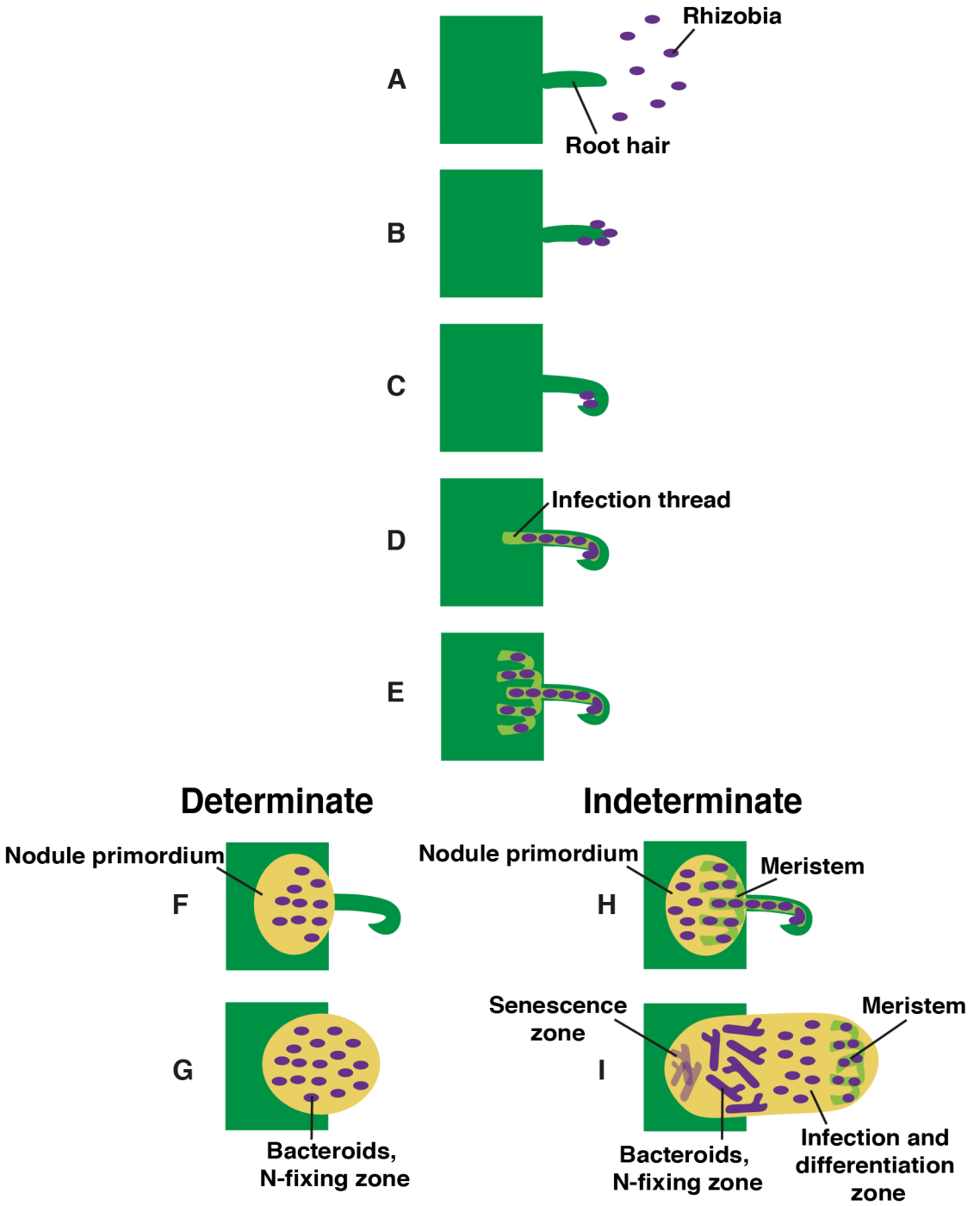


Figure 1.1 – Nodule formation in the two main nodule types. (A) Roots produce flavonoids which attract rhizobia, inducing Nod factor production. (B) Rhizobia attach to root hairs. (C) The root hair curls, trapping rhizobia. (D) An infection thread forms by plant cell wall invagination, which rhizobia grow through into the root. (E) Infection thread progresses through outer cortex and inner cortex of root, growing towards the nodule primordium. (F) In developing determinate nodules, rhizobia are released into the nodule primordium and begin differentiating into bacteroids. (G) The mature determinate nodule has a homogenous structure, with no maintained meristem, where all rhizobia have differentiated into bacteroids and fix nitrogen. (H) In developing indeterminate nodules, a meristem is maintained, and rhizobia are released from the infection thread into the nodule primordium. (I) In mature indeterminate nodules distinct zones are observed. Bacteria are maintained in the meristem, from which they are released to infect the nodule and differentiate into bacteroids, bacteroids fix nitrogen in the N-fixing zone, and older plant cells and bacteroids senesce in the senescence zone.

Recognition of Nod factors by the plant triggers an internal cascade, of which many components share a common pathway with those regulating the arbuscular mycorrhizal symbiosis[41]. Nod factor detection stimulates calcium oscillations in the nucleus[50], triggering a protein kinase to activate nodule signalling pathway transcription factors[50,51]. These transcription factors activate a downstream pathway resulting in nodule and infection thread formation[52]. The rhizobia must then attach to and infect the root. In some legume species, root infections occur directly into the cortex via the root surface or the base of emerging roots[53]. More commonly, rhizobia attach to a root hair causing inward curling, which enables them to pass through the root cortex in an infection thread[40]. Rhizobia grow along this infection thread, where host specificity can be further imposed through receptor-like kinases[54]. When the infection thread reaches the nodule primordium, the rhizobia enter the plant cells by endocytosis and are enclosed by a symbiosome membrane[55,56]. The rhizobia can then differentiate into nitrogen-fixing bacteroids[39], as the low-oxygen environment of the nodule stimulates the expression of nitrogen-fixation genes[55,57,58].

1.3.3 Nodule types

There are two main nodule types in the *Fabaceae*, and the type of nodule formed is dependent on the host plant[59,60]. Legumes of the non-galegoid clade such as *Phaseolus* and *Glycine* have determinate nodules, which are spherical and do not continue to grow[39]. These nodules do not maintain a meristem, and all rhizobia differentiate into bacteroids which are of similar size to the free-living cells, fix nitrogen, and can survive and reproduce outside of the nodule[56,60–62].

The second main nodule type is an indeterminate nodule, as seen in legumes of the galegoid clade such as *Pisum*, *Medicago* and *Vicia*[56]. These nodules maintain a persistent meristem and so can continue to grow, resulting in elongated or branched nodules[39,55], and here rhizobia are dimorphic. Some rhizobia remain as bacteria in the meristem, which do not fix nitrogen but retain the ability to reproduce. Alternatively, some rhizobia terminally differentiate into larger, nitrogen-fixing bacteroids by undergoing chromosomal endoreduplication, which causes them to lose their ability to reproduce[56,60–63]. Endoreduplication and terminal differentiation is driven by nodule-specific cysteine-rich (NCR) peptides[64], which are expressed by the host plant in bacteroid-containing cells and can cause transcriptional changes of genes involved in cell division[65].

1.3.4 Nutrient exchange

Within the nodule, rhizobia are housed within symbiosome membranes across which nutrient exchange occurs between the plant and the rhizobia[56]. Carbon is provided to rhizobia as dicarboxylic acids which are transported across the bacteroid and symbiosome membranes[66,67] and utilised for respiration, nitrogen fixation, and the production of carbon storage molecules[68]. Nitrogen fixation is carried out by the nitrogenase enzyme, which converts dinitrogen gas to ammonia[14]. This enzyme is oxygen sensitive, so nodule legheamoglobins bind to oxygen, reducing the overall oxygen concentration to maintain nitrogenase function, while delivering oxygen to the bacterial cells when needed for respiration[14,69]. The resultant ammonia is transported out of the symbiosome and assimilated into amino acids by the host plant[14]. Nodule rhizobia are also reliant on their plant hosts for the provision of amino acids[70] and ions such as sulfate[71], phosphate[72], and various metal ions[14].

1.4 Host regulation of the legume-rhizobia symbiosis

1.4.1 Gains and losses of the symbiosis

Despite disputes over the precise number of origins of the nitrogen-fixing symbiosis, it is generally agreed that the nitrogen-fixing symbiosis has been lost multiple times throughout the nitrogen-fixing clade[30,33–37]. This suggests that under certain conditions, the benefits a plant obtains from the symbiosis no longer outweigh the costs of investing in the system.

There are three main possible drivers for the loss of the symbiosis. First, some species may be able to obtain sufficient nitrogen from the soil. However, this is unlikely to have affected such a wide range of species, as nitrogen is a limiting factor in most ecosystems[73]. The second cause might arise when plant species colonise new environments. A meta-analysis of fixing and non-fixing legume species showed that non-fixing legumes were more successful at establishing new populations outside of their native range[38]. This suggests that the dependence of fixing strains on locale-specific bacterial partners can be detrimental and restrict species' ranges. The third possible cause for loss of the symbiosis could occur when bacterial strains evolve to fix so little nitrogen that they are not worth the investment of carbon from the plant[74]; i.e. that the symbiosis breaks down.

Rhizobial strains which provide little or no nitrogen for the host plant do exist. Rhizobia with a wide range of fixation abilities have been isolated from host plants in the field[27,75,76] and poor-fixers might be expected because rhizobia are not vertically transmitted. Instead they must be newly acquired from the soil in each generation, which leads to a de-coupling of the fitness interests of host and symbiont[77]. As most plants in the field are

simultaneously infected by multiple rhizobial strains[78–81], a non-fixing or poorly-fixing rhizobia could then exploit the host by receiving nutrients, but relying on other strains to provide the host with nitrogen[82]. This could increase the fitness of the rhizobia, as it avoids the high energetic cost of fixing nitrogen[82] and instead can invest more in reproduction[83,84], while decreasing the fitness of the host plant. This potential conflict between partners could cause the breakdown of the symbiosis between rhizobia and their hosts over evolutionary time[85,86].

However, plants are not completely defenceless against cheating strains. To prevent wasting resources on non-fixing rhizobia, the plant has two potential options: partner choice and sanctioning, although sometimes these terms are used interchangeably[86–89]. Partner choice refers to the screening of potential partners for their ability to provide benefits before a mutualistic relationship has started[77,90–95]. In the case of the legume-rhizobia symbiosis, this would result in the host plant forming fewer nodules with less effective strains. In contrast, sanctioning refers to the differential treatment of partners that provide different benefits once the relationship has begun[74,77,89,90,92–97]. In the case of the legume-rhizobia symbiosis, this would result in a fitness cost to the rhizobia, where fewer viable rhizobia remain inside the nodule.

Both of these mechanisms would prevent the plant wasting resources on strains which provide little or no nitrogen[84], though partner choice may be more effective at this, as even more resources are spared by not initiating nodule formation. These mechanisms may also prevent the spread of ineffective strains through the population. By not forming a nodule, or by being sanctioned within a nodule, a strain does not get the fitness benefits and population increase that comes from forming a healthy nodule[74,77,90].

1.4.2 Partner choice

Partner choice, or forming fewer nodules with less effective strains, has been demonstrated in several host plant species[77,89,91,94,95,98,99]. Some of these studies have shown partner choice to be acting alone[77,94], and others in combination with sanctioning[91,95]. However, these studies all used naturally-occurring strains of rhizobia which were collected from the field, which may differ in several traits which affect their ability to form nodules[100], such as growth rate[91], motility[101], and root attachment[102]. It is therefore unclear from these studies whether the observed partner choice was a result of differences in nitrogen-fixation between strains, or differences in other characteristics.

Some observations of partner choice may also be due to the host range specificity of the rhizobia, rather than the plant preferentially choosing strains based on effectiveness. Some rhizobia have a narrow host range, such as *Rhizobium leguminosarum* bv. *trifolii* and *Sinorhizobium meliloti*, whose host plants are limited to one or two genera, respectively[1,103]. Alternatively, some rhizobia have a wide host range, such *Sinorhizobium fredii* strains USDA257 and NGR234, which are reported to be able to form nodules with 79 and 112 host plant genera, respectively[1,59]. This specificity is enforced by the complex signalling system involved in nodule formation and development, including flavonoids, Nod factors, effector-triggered immunity and polysaccharides[43,48,49]. These signals mean host plants are likely to only form nodules with rhizobia which are likely to be well-adapted to that host. However, these signals do not necessarily indicate the effectiveness of a strain, as mutations could arise which alter the fixation ability of a strain without altering its signalling mechanisms.

It remains unclear how the host plant could assess differences in nitrogen-fixation ability before nodules are formed. It is possible that differences in nitrogen-fixation genes between rhizobial strains could indicate differences in nitrogen-fixation ability, but these genes are only expressed under the low oxygen concentrations within a nodule[55,57,58]. Large-scale mutagenesis and sequencing suggests these genes are only important inside the nodule, not for root colonisation[104]. Additionally, despite partner choice being observed in multiple studies[77,89,91,94,95,98,99], none were able to link partner choice to differences in nitrogen-fixing, or other, rhizobial genes. Therefore, there is currently no evidence of the indicator which host plants use to assess nitrogen fixation rates before a nodule is formed.

Study of strains which differ in their nitrogen-fixation abilities alone would give better insight into whether hosts can choose their partners based on this characteristic. Such a test was conducted by Westhoek *et al.* (2017)[105], who showed that pea plants (*Pisum sativum*) did not preferentially form nodules with a fixing strain, compared to a non-fixing mutant. However, nodules of the non-fixing mutant were smaller than nodules of the fixing strain[105], suggesting that hosts can still sanction strains based only on their nitrogen-fixation abilities.

1.4.3 Sanctioning partners

As mentioned above, host plants can also prevent wasting resources on ineffective strains by sanctioning such strains: cutting off resources once they have formed a nodule and their nitrogen fixing ability has become apparent. Several studies have shown that plants can sanction strains which differ in only their nitrogen fixing ability. This has been

achieved by withholding nitrogen gas to reduce nitrogen fixation[90,93,106], or by genetically altering the fixation ability of a strain[105,107,108].

Kiers *et al.* (2003), conducted the first such study withholding nitrogen gas using *Bradyrhizobium japonicum* and the host plant soybean (*Glycine max*). Here, individual nodules, half of a split-root, or the whole plant root, was enclosed in a chamber and supplied with a mixture of nitrogen and oxygen as a control, or argon and oxygen to prevent nitrogen fixation. In all systems the non-fixing nodules had fewer viable rhizobia per nodule than fixing nodules. Additionally, non-fixing nodules had reduced fresh weights compared to fixing nodules in the split-root and individual nodule systems, consistent with the hypothesis of sanctioning[90]. Similar results were seen in split-root experiments by Oono *et al.* (2011), using *Rhizobium leguminosarum* with the host plant pea (*Pisum sativum*), and *Sinorhizobium meliloti* with the host plant alfalfa (*Medicago sativa*)[93]. Further work by Kiers *et al.* (2006) provided differing amounts of nitrogen gas to nodules, forcing a range of fixation rates. Here the fresh weights and number of viable rhizobia per nodule was proportional to the fixation rate of the nodule, suggesting that plants can sanction strains in proportion to their fixation ability[106].

Similar observations of sanctioning are seen in strains which have been genetically altered to be unable to fix nitrogen. Westhoek et al. (2017) used *Rhizobium leguminosarum* with the host plant pea (*Pisum sativum*), and competed a fixing strain against a non-fixing *nifH* mutant[105], a key gene in nitrogen fixation[57]. Here nodules containing the non-fixing *nifH* mutant were smaller than nodules containing the fixing strain, supporting the hypothesis of sanctioning. Daubech *et al.* (2017) also studied a non-fixing *nifH* mutant, this time using *Cupriavidus taiwanensis* with the host plant *Mimosa pudica*. Here, non-fixing

strains had fewer viable rhizobia per nodule than fixing strains, again supporting the hypothesis of sanctioning[107]. As the strains in these studies are otherwise isogenic, any differences seen in the effect of host sanctions between fixing and non-fixing strains can be attributed to their nitrogen fixation rate, rather than other characteristics.

1.4.4 Sanctioning within a mixed nodule

Sanctioning has been demonstrated at the whole nodule level, and whilst the majority of nodules observed in the lab and field contain just one strain[109], mixed nodules can form, which can contain two or more strains[96,105,107,110–114]. Recent work by Mendoza-Suárez *et al.* (2020) found up to six different strains in some *Pisum sativum* nodules[113]. Mixed nodules are most likely to occur when one strain signals to the plant, and others are also trapped in the curling root hair, resulting in two or more strains in the infection thread[109]. The frequency of mixed nodules can depend on the density of rhizobia available to the plant[105], but has been observed to range from 2% to all nodules depending on the host[97,107,111,113]. Therefore, the ability to sanction *within* a mixed nodule, reducing the fitness of one strain but not the other, could give the host plant further control of the distribution of resources to ineffective strains. Determining whether hosts can sanction within mixed nodules would also give clues to the cue that the host plant relies on to be able to discriminate between strains.

The effectiveness of sanctions to maintain cooperation in symbioses depends on the ability of the host to compartmentalise its symbionts[115]. Given the prevalence of mixed nodules, understanding if and how sanctioning acts within mixed nodules is important for understanding the long-term dynamics of rhizobial populations. If hosts cannot discriminate between the fixation rates of two strains within a mixed nodule and sanction

accordingly, then sharing a nodule with a fixing strain could allow a non-fixing strain to avoid sanctioning[77,97]. If the fixing strain provides enough nitrogen to avoid being sanctioned by the host plant, then the non-fixing strain could gain higher fitness through investing in reproduction[83,84].

Studies of sanctioning within mixed nodules is limited, and the results contrasting. Checcucci *et al*. (2016) published the first study of sanctioning within a mixed nodule, using *Sinorhizobium meliloti* and the host plant *Medicago sativa*, which forms indeterminate nodules. Here, there was no conclusive evidence of sanctioning within a mixed nodule. The non-fixing strain had similar numbers of viable rhizobia per mixed nodule compared to the fixing strain[111]. Contrastingly, Daubech *et al*. (2017) showed evidence of sanctioning within a mixed indeterminate nodule using *Cupriavidus taiwanensis* and the host plant *Mimosa pudica*. Here a fixing strain had more viable rhizobia per mixed nodule than an otherwise isogenic non-fixing strain[107]. The discrepancy between these two studies could be due to the use of different host plants, as perhaps sanctions are not uniform across hosts. However, the age of the nodules selected may also play a role in the differing results: Daubech *et al.* (2017) only saw evidence of sanctioning within a mixed nodule at 35 dpi (days post inoculation) and not at 14 dpi[107], whilst Checcucci *et al*. (2016) used a range of nodule ages starting from just 10 dpi[111].

Evidence also suggests that sanctioning within mixed nodules can affect bacteroids. Daubech *et al.* (2017) used confocal microscopy to demonstrate that within mixed nodules the non-fixing strain formed bacteroids which looked healthy at 14 dpi, but were senescing by 35 dpi[107]. This is in line with a study by Regus *et al.* (2017) using *Mesorhizobium loti* with the determinate host *Lotus japonicus*. Here confocal microscopy

also showed that bacteroids of the non-fixing strain appeared to be undergoing senesce at 49 dpi, but not as early as 28 dpi[96]. These studies show that sanctioning within a mixed nodule has only been observed at a later timepoint in nodule development, but further studies across a variety of hosts, with both nodule types, could confirm this phenomenon.

1.4.5 Mechanisms of sanctioning

Although sanctioning has been observed frequently in the legume-rhizobia symbiosis, there is little understanding of how the host plant regulates sanctioning, and no host plant genes involved in the process have yet been identified. Plants can limit the number of nodules they form based on nitrate availability and the number of existing nodules, known as autoregulation[39,116–118]. This suggests that the plant can compare cues of nitrate status for nodules and the whole root system in order to regulate nodulation. The cue can likely travel over the whole root system, as Lambert *et al.* (2020) showed that nodules on one split root half can be senesced after the provision of external nitrate to the other half[119].

Kiers *et al.* (2003) showed that not only can sanctioning act at the level of the whole root system, but can also be targeted to one half of a split-root, or to an individual nodule[90], which has been corroborated by further studies[93,105–107]. This suggests that plants can respond to the nitrogen-fixing abilities of individual nodules. Furthermore, Daubech *et al.* (2017) and Regus *et al.* (2017) showed that sanctioning can also act at a within-nodule level[96,107], suggesting that plants can respond to the nitrogen-fixing abilities of individual strains within a nodule.

Despite these advances, the identity of the cue, or cues, which plants use to compare nodules have yet to be identified. Additionally, whether plants can sanction strains based

on their relative fixation ability compared to the other available strains is less clear. Kiers *et al.* (2006) showed that intermediate fixers are sanctioned in proportion to their fixation levels[106]. However, this study did not investigate a situation where the intermediate fixer was the best available option for the plant. Therefore, it is unknown whether plants can compare all available nodules, or strains within nodules, and only sanction a strain when a better option is available.

Similarly to the lack of knowledge about the cues involved in sanctioning, it is also not known by which mechanism nodules, or rhizobia within nodules, are sanctioned. Postulated mechanisms include the host detection of a poorly-fixing nodule by signalling[83] and the subsequent reduction of oxygen, amino acid or nutrient supply to the nodule, leading to nodule senescence[70,90,96,108]. Alternatively, the observed sanctioning effect could be due to metabolic fluxes. Nitrogen-fixing nodules produce ammonia, requiring a large inwards carbon flux to convert this ammonia into amino acids and urides[14]. Non-fixing nodules produce less ammonia, so may have a smaller inwards carbon flux, and therefore a reduced metabolic capacity for other activities such as reproduction, which could result in smaller nodules containing fewer bacteria.

1.4.6 Differences in regulation between nodule types

The effects of partner choice and sanctioning become more complex when the differences between determinate and indeterminate nodules are taken into account. As described earlier, inside determinate nodules, once nodules are formed, rhizobia differentiate into bacteroids which are of a similar size to free-living cells and retain their ability to reproduce[56,60–62]. In contrast, inside indeterminate nodules, rhizobia are dimorphic: they either do not fix nitrogen and retain their ability to reproduce; or they terminally

differentiate into larger non-reproductive nitrogen-fixing bacteroids[56,60–63]. Therefore, while all rhizobia in determinate nodules can both reproduce and fix nitrogen, rhizobia in indeterminate nodules can *either* reproduce *or* fix nitrogen.

If legumes are capable of partner choice, then this too could potentially differ between nodule types, due to differences in the signalling mechanisms required for nodule formation[49]. Additionally, while not a mechanism for pre-infection partner choice, in indeterminate nodules the host produces NCR peptides to induce endoreduplication and differentiation of rhizobia into bacteroids[64,65]. These peptides are detected by the rhizobia using various membrane proteins, such as BacA and BclA[120–123]. Rhizobia that are deficient in BacA and BclA cannot respond to NCR peptides and therefore cannot differentiate, and undergo premature senescence once released into the nodule[120,121,123]. However, *bacA* mutants are still able to form healthy nodules with determinate hosts[124]. This suggests that NCR peptides are crucial for the formation of indeterminate nodules, and that this system gives indeterminate hosts a further level of control over which rhizobial partners are invested in.

The outcome of sanctioning could also hypothetically differ between the two nodule types, depending on whether sanctioning impacts only bacteroids or whether it also impacts their undifferentiated clonemates. In determinate nodules, sanctions targeted at the bacteroids will also directly reduce rhizobial fitness, as the bacteroids retain their ability to reproduce[125]. Evidence of this is provided by Kiers *et al.* (2003, 2006)[90,106], using *Bradyrhizobium japonicum*, which forms determinate nodules with soybean (*Glycine max*). As described earlier, nodules were enclosed in a chamber and nitrogen gas replaced with argon, which reduced or prevented nitrogen fixation, and so forced the

rhizobia to cheat on the plant. In response, the plant sanctioned the non-fixing or poorly-fixing nodules, and these nodules were found to contain fewer viable rhizobia. This shows that sanctioning can reduce the fitness of rhizobia in determinate nodules.

In indeterminate nodules, sanctions directed only at bacteroids would prevent the host wasting resources on ineffective strains[74], but it may not affect the fitness of their reproductive clonemates[93,125] which are maintained by a persistent meristem[39,55]. This means that sanctioning in plants with indeterminate nodules may still allow non-fixing strains to prosper. However, Oono *et al*. (2011)[93] was the first to show a reduction in the number of undifferentiated bacteria for non-fixing nodules in two hosts which form indeterminate nodules (*Pisum sativum* with *Rhizobium leguminosarum*, and *Medicago sativa* with *Sinorhizobium meliloti*). Further studies by Daubech *et al*. (2017)[107] and Westhoek *et al.* (2021)[108] have similarly shown sanctioning in indeterminate hosts to affect the number of undifferentiated bacteria. It therefore appears that sanctioning is not just aimed at bacteroids, but can also reduce bacterial fitness.

As sanctioning has been shown to affect the number of reproductive rhizobia per nodule in both nodule types, then sanctioning could reduce the population size of poorly-fixing rhizobia in comparison to well-fixing rhizobia, potentially leading to their eradication from the soil population[107]. Given the differences between the internal structures of the two nodules, comparative studies could reveal whether the same mechanisms of sanctioning are seen across both nodule types, and also across multiple legume hosts.

1.5 Research objectives

This thesis aimed to further characterise the ways in which host plants can regulate their symbiosis with nitrogen-fixing rhizobia. Using the host plant pea (*Pisum sativum*), and otherwise isogenic fixing, intermediate-fixing and non-fixing derivatives of the standard laboratory rhizobial strain *Rhizobium leguminosarum* bv. *viciae* 3841, we investigated whether the host plant can compare and discriminate between the different rhizobial strains which are available. We looked at mixed nodules which contain more than one strain, to see if the host can discriminate between strains at a within-nodule level, and sanction accordingly. Finally, we expanded this work across the two main nodule types in the legume-rhizobia symbiosis, to compare the mechanisms of host regulation. Here we used pea (*Pisum sativum*, indeterminate nodules) and bean (*Phaseolus vulgaris*, determinate nodules) with otherwise isogenic fixing and non-fixing strains of *R. leguminosarum.*

1.5.1 Chapter 3: Conditional sanctioning of an intermediate fixer

In **Chapter 3** we investigated how a host plant allocates resources to a strain which fixes an intermediate amount of nitrogen. Studies show that hosts can sanction nodules containing non-fixing strains[90,93,94,105–108,126]. Additionally, when only one strain is available, the host can proportionally sanction this strain based on its fixation level[106]. Here we determined whether the host plant can compare the strains in its nodules and sanction accordingly. Specifically, if a host can sanction an intermediate-fixing strain when it is the worst available option, which we term conditional sanctioning[108]. We assessed how conditional sanctioning affects the size of the nodule, the amount of carbon being supplied to the nodule, the nitrogen-fixation rate of the nodule, and the number of

viable rhizobia in the nodule. Findings from this chapter were combined with modelling work of another researcher to form a published manuscript[108].

1.5.2 Chapter 4: Quantifying sanctioning at a single-cell level using flow cytometry

In **Chapter 4** we investigated the effects of sanctioning within a nodule, at a single-cell level. Studies have shown sanctioning to reduce the number of viable rhizobia per nodule[93,94,105,107,108,126]. However for indeterminate nodules, only the viable bacteria can be cultured and counted in this way, not the terminally differentiated nitrogen-fixing bacteroids[55,56,60–63]. Histological studies show fewer bacteroids[126] and reduced bacteroid senescence[107] in less effective nodules, but the effect of sanctioning on the number of bacteroids has not yet been quantified. Here we optimised the use of flow cytometry, a single-cell analysis technique, to test whether sanctioning of a non-fixer, or an intermediate-fixer, results in changes to the numbers of bacteroids per nodule. Given mixed evidence for the ability of host plants to sanction within a mixed nodule[96,107,111], we determined whether hosts could sanction a non-fixer or an intermediate-fixer within a mixed nodule. We assessed this by comparing the number of reproductive rhizobia which can be extracted from a nodule, and again using flow cytometry to determine the number of bacteria and bacteroids within a nodule.

1.5.3 Chapter 5: partner choice and sanctioning in determinate and indeterminate nodules

In **Chapter 5** we compared the mechanisms of host regulation between the two main nodule types in the symbiosis, determinate and indeterminate nodules. Host plants could regulate non-fixing strains by screening them out before they enter nodules (partner choice)[77,90–95] or by differential treatment of strains that provide different benefits once

the nodule has formed (sanctioning)[74,77,89,90,92–97]. This may differ between the two nodule types given the different bacterial life-histories within these nodules[61]. Indeterminate hosts have been observed to exert partner choice[77,94,98] and sanction nodules[93,107], but when strains differ only in their nitrogen fixation ability, plants can sanction nodules, but not exert partner choice[105,107]. Evidence of partner choice[89,91,95,99,127] and sanctioning[90,96,106] is also shown in determinate hosts, but no definitive test of sanctioning and partner choice has been carried out in determinate hosts for strains which differ only in their nitrogen-fixing ability. Here we tested whether a host with each nodule type can exert partner choice over or sanction its rhizobial partners. To test for partner choice, we assessed whether host plants form proportionally more nodules with fixing strains compared to non-fixing strains. To test for sanctioning, we assessed whether nodules containing fixing strains are larger than those containing non-fixing strains. We also compared the size of fixing nodules when they were present at different frequencies, to see if the host can regulate investment into these nodules depending on how rare they are.

Chapter 2 General methods

2.1 Overview

This chapter contains details of the general methods used in this thesis. The experimental designs, methods which differ between each experiment, and the statistical tests used, are detailed individually for each experiment in **Chapter 3**, **Chapter 4,** and **Chapter 5**.

2.2 Media, antibiotics, and other chemicals

2.2.1 Media

Escherichia coli strains were grown in sterile Luria Bertani (LB) broth (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 5 g L^{-1} NaCl)[128]. For solid media, 14 g L^{-1} agar was added. *Rhizobium leguminosarum* strains were grown in sterile tryptone yeast (TY) media (3 g L^{-1} yeast extract, 5 g L^{-1} tryptone, 1.3 g L^{-1} $CaCl_2$)[129]. For solid media, 17.5 g L^{-1} agar was added. Seeds were germinated on 0.8% sterile distilled water agar (DWA) (8 g L^{-1} agar in distilled H_2O).

2.2.2 Antibiotics

Antibiotics for selection were added to media in the concentrations shown in **Table 2.1**.

Antibiotic	**Abbreviation**	**Working concentration µg mL^{-1}**	
		E. coli	***R. leguminosarum***
Ampicillin	Amp	100	-
Carbenicillin	Cb	50	-
Gentamicin	Gm	10	20
Kanamycin	Km	20	-
Neomycin	Nm	-	40
Nitrofurantoin	Nt	-	10
Rifampicin	Rf	-	50
Spectinomycin	Sp	50	100
Streptomycin	St	-	500
Tetracycline	Tc	10	5

Table 2.1 – Working concentrations of antibiotics used for strain selection.

2.2.3 Other chemicals

Growth media was supplemented with additional chemicals for selection or to support the growth of auxotrophic strains. 40 μg mL^{-1} X-gal was added to LB media for blue-white selection of transformants[130,131]. *E. coli* ST18 strains (used to carry plasmids) were grown on LB supplemented with 50 μg mL^{-1} 5-aminolevulinic acid (ALA) as ST18 cells are ALA auxotrophs[132]. Extracts from nodules were plated on media supplemented with 50 μg mL^{-1} nystatin to prevent fungal growth.

2.3 Bacterial strains, plasmids, and primers

2.3.1 Bacterial strains

The strains used in this thesis are detailed in **Table 2.2**.

Strain	Description	Antibiotic resistance	Reference
***E. coli* strains**			
α-Select Gold	Competent strain for use in transformations, carrying the following mutations: *F- deoR endA1 recA1 relA1 gyrA96 hsdR17(rk -, mk+) supE44 thi-1 phoA Δ(lacZYA argF)U169 Φ80lacZΔM15λ-*	-	Bioline
DH5α	Competent strain for use in transformations, carrying the following mutations: *F- Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17(rk-, mk+) phoA supE44 thi-1 gyrA96 relA1 λ-*	-	Invitrogen
EC100D ™ pir-116	Competent strain for use in transformations, carrying the following mutations: *F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ- rpsL (Str^R) nupG pir-116(DHFR)*	-	Lucigen
ST18	Mating strain for use as donor in conjugations, S17 Δ*pir* Δ*hemA*	(ALA)	[132]
***R. leguminosarum* strains**			
LMB640	RU3940[pJP2] (Rlv3841 *nifH*::Ω Sp^r with pJP2)	St, Sp, Tc	[133]
OPS0365	Rlv3841 *gusA nifH*::Ω Sp^r	St, Sp	[105]
OPS0366	Rlv3841 *celB nifH*::Ω Sp^r	St, Sp	[105]
OPS1339	Rlv3841 with pOPS0743 used to insert sfGFP under J23104 promoter (Tn*7*)	St, Gm	[108]

OPS1341	Rlv3841 with pOPS1015 used to insert mCherry3 under J23104 promoter (Tn*7*)	St, Gm	[108]
OPS1457	Rlp4292 *nifK*::Ω Sp^r, non-fixing phenotype	Rf, Sp	This work
OPS1474	Ω Sp^r resistant insertion between *nifA* and *fixX* of Rlv3841, intermediate fixation phenotype (previously named LMB827)	St, Sp	[108]
OPS1475	Rlv3841 *fixC*::Ω Sp^r, non-fixing phenotype	St, Sp	This work
OPS1480	RlvA34 *nifK*::Ω Sp^r, non-fixing phenotype	St, Sp	This work
OPS2136	OPS1474[pLMB714] (intermediatefixer with C4-dicarboxylates biosensor) (triparental)	St, Sp, Tc	[108]
OPS2137	OPS1474[pLMB687] (intermediatefixer with sucrose biosensor) (triparental)	St, Sp, Tc	[108]
OPS2268	OPS1474 (intermediatefixer) with pOPS1526 used to insert sfGFP under J23104 promoter (Tn*7*)	St, Sp, Gm	[108]
OPS2269	OPS1474 (intermediatefixer) with pOPS1531 used to insert mCherry3 under J23104 promoter (Tn*7*)	St, Sp, Gm	[108]
OPS2270	RU3940 (Rlv3841 *nifH*::Ω Sp^r) with pOPS1526 used to insert sfGFP under J23104 promoter (Tn*7*)	St, Sp, Gm	[108]
OPS2271	RU3940 (Rlv3841 *nifH*::Ω Sp^r) with pOPS1531 used to insert mCherry3 under J23104 promoter (Tn*7*)	St, Sp, Gm	[108]
OPS2272	RU1416 (Rlv3841[pJP2]) with pOPS1531 used to insert mCherry3 under J23104 promoter (Tn*7*)	St, Gm, Tc	[108]
OPS2273	LMB640 (Rlv3841 *nifH*::Ω Sp^r with pJP2) with pOPS1531 used to insert mCherry3 under J23104 promoter (Tn*7*)	St, Sp, Gm, Tc	[108]
OPS2278	OPS2137 (intermediatefixer with sucrose biosensor) with pOPS1526 used to insert sfGFP under J23104 promoter (Tn*7*)	St, Sp, Gm, Tc	[108]
OPS2306	RlvA34 with pOPS1548 used to insert *gusA* under Ps*nifH* promoter (Tn*7*)	St, Gm	This work
OPS2307	RlvA34 with pOPS1549 used to insert *celB* under Ps*nifH* promoter (Tn*7*)	St, Gm	This work
OPS2308	OPS1480 (RlvA34 *nifK*::Ω Sp^r) with pOPS1548 used to insert *gusA* under Ps*nifH* promoter (Tn*7*)	St, Sp, Gm	This work
OPS2309	OPS1480 (RlvA34 *nifK*::Ω Sp^r) with pOPS1549 used to insert *celB* under Ps*nifH* promoter (Tn*7*)	St, Sp, Gm	This work
OPS2313	OPS2136 (intermediatefixer with C4-dicarboxylates biosensor) with pOPS1526 used to insert sfGFP under J23104 promoter (Tn*7*)	St, Sp, Gm, Tc	[108]
OPS2314	OPS2268[pLMB640] (intermediatefixer with pOPS1526 used to insert sfGFP under J23104 promoter and with P*nifH* biosensor) (triparental)	St, Sp, Gm, Tc	[108]
OPS2315	OPS2269[pJP2] (intermediatefixer with pOPS1531 used to insert mCherry3 under J23104 promoter and with pJP2) (triparental)	St, Sp, Gm, Tc	This work
OPS2348	Rlp4292 with pOPS1549 used to insert *celB* under Ps*nifH* promoter (Tn*7*)	Rf, Gm	This work

OPS2349	OPS1457 (Rlp4292 *nifK*::Ω Sp^r) with pOPS1549 used to insert *celB* under Ps*nifH* promoter (Tn*7*)	Rf, Sp, Gm	This work
OPS2364	OPS2268[pIJ11282] (intermediatefixer with pOPS1526 used to insert sfGFP under J23104 promoter and with P*neo* biosensor) (triparental)	St, Sp, Gm, Tc	This work
OPS2373	Rlp4292 with pOPS1548 used to insert *gusA* under Ps*nifH* promoter (Tn*7*)	Rf, Gm	This work
OPS2374	OPS1457 (Rlp4292 *nifK*::Ω Sp^r) with pOPS1548 used to insert *gusA* under Ps*nifH* promoter (Tn*7*)	Rf, Sp, Gm	This work
Rlp4292	Rf^r derivative of *R. leguminosarum* bv. *phaseoli* strain 8002	Rf	[134]
Rlv3841	St^r derivative of *R. leguminosarum* bv. *viciae* strain 300	St	[110]
Rlv3841 *gusA*	Rlv3841 with *gusA* marker	St	[135]
RlvA34	St^r derivative of *R. leguminosarum* bv. *phaseoli* strain 8002, with loss of pRP2 Sym plasmid and addition of pRL1 Sym plasmid from *R. leguminosarum* bv. *viciae* 248	St	[136]
RU1416	Rlv3841[pJP2]	St, Tc	[137]
RU3940	Rlv3841 *nifH*::Ω Sp^r, non-fixing phenotype	St, Sp	[105]

Table 2.2 – Strains used in this thesis. (Tn7) and (triparental) indicate the marking method used.

2.3.2 Plasmids

The plasmids used in this thesis are detailed in **Table 2.3**. Plasmids were maintained in *E. coli* cells.

Plasmid	Description	Antibiotic resistance	Reference
pDAI-SceI	*Sce*I homing endonuclease expressing plasmid for counterselection	Tc	[138]
pHP45Ω-Sp	pRB322 derivative containing ΩSp^r cassette	Sp, Amp	[139]
pIJ11268	Broad host range plasmid; promoterless *luxCDABE* derived from pJP2	Amp, Tc	[140]
pIJ11282	Constitutive P*neo* biosensor, pIJ11268 with *nptII* promoter cloned in front of *luxCDABE*	Tc	[140]
pJET1.2	Blunt cloning vector derived from pUC19	Amp	Thermo Fisher
pJP2	Stable broad host range cloning vector	Tc	[141]
pJQ200SK	Suicide vector for cloning, pACYC derivative, P15A origin of replication; *lacZ sacB traJ*	Gm	[142]
pLMB640	P*nifH* biosensor, pIJ11268 with *nifH* promoter cloned in front of *luxCDABE*	Tc	[108]

pLMB687	Sucrose biosensor, pIJ11268 with sucrose transporter promoter cloned in front of *luxCDABE*	Tc	[143]
pLMB714	C4-dicarboxylates biosensor, pIJ11268 with *dctA* promoter cloned in front of *luxCDABE*	Tc	[143]
pLMB878	Rlv3841 *fixC::Ω* Sp^r in pJQ200SK	Gm, Sp	[108]
pOGG003	pL0M-T-pharma. Level 0 T module for Golden Gate cloning	Sp	Invitrogen
pOGG026	pL1V-Lv1-neo-RK2-par-ELT4, 6.6 kb, low copy, stable, broad-host range Level 1 vector for Golden Gate cloning	Km/Nm	[144]
pOGG043	pL0M-PU-Ps*nifH*. Level 0 PU module for Golden Gate cloning	Sp	[113]
pOGG050	pL0M-SC-*celB*. Level 0 SC module for Golden Gate cloning	Sp	[144]
pOGG083	pL0M-SC-*gusA*. Level 0 SC module for Golden Gate cloning	Sp	[144]
pOGG129	pL0M-SC-mTagBFP[145]. Level 0 SC module for Golden Gate cloning	Sp	[146]
pOGG157	pL0M-T-DT16. Level 0 T module for Golden Gate cloning	Sp	[146]
pOGG276	pL1V-Lv1-pUC18R6K-miniTn*7*T-Gm, mobilisable broad-host range Level 1 vector for Golden Gate cloning, for Tn*7* insertion	Amp, Gm	[147]
pOPS0743*	sfGFP under J23104 promoter in a pUC18T-miniTn*7*T-Gm vector for Tn*7* insertion	Amp, Gm (ALA)	[108]
pOPS0997*	Rlp4292 *nifK*::Ω Sp^r in pJQ200SK	Sp, Gm (ALA)	This work
pOPS0998*	RlvA34 *nifK*::Ω Sp^r in pJQ200SK	Sp, Gm (ALA)	This work
pOPS1015*	mCherry3 under J23104 promoter in a pUC18T-miniTn*7*T-Gm vector for Tn*7* insertion	Amp, Gm (ALA)	[108]
pOPS1146	Reporter plasmid constructed with Ps*nifH* (pOGG043), mTagBFP (pOGG129) and T-pharma (pOGG003) assembled in pOGG026	Km/Nm	This work
pOPS1526*	sfGFP under J23104 promoter in a pUC18R6KT-miniTn*7*T-Gm vector for Tn*7* insertion	Amp, Gm (ALA)	[108]
pOPS1531*	mCherry3 under J23104 promoter in a pUC18R6KT-miniTn*7*T-Gm vector for Tn*7* insertion	Amp, Gm (ALA)	[108]
pOPS1548*	*gusA* under Ps*nifH* promoter in a pUC18R6KT-miniTn*7*T-Gm vector for Tn*7* insertion	Amp, Gm (ALA)	This work
pOPS1549*	*celB* under Ps*nifH* promoter in a pUC18R6KT-miniTn*7*T-Gm vector for Tn*7* insertion	Amp, Gm (ALA)	This work
pRK2013	Helper plasmid for triparental conjugation	Km	[148]
pTNS3*	Plasmid expressing *tnsABCD* from *P1* and P_{lac}	Amp (ALA)	[149]

Table 2.3 – Plasmids used in this thesis. * indicate plasmids which are in also available in ST18 backgrounds, and therefore require supplementation with ALA when this background is used.

2.3.3 Primers

The primers used in thesis are detailed in **Table 2.4**. Primers were synthesised by Eurofins Genomics.

Primer	Sequence (5' to 3')	Description
515F	GTGCCAGCMGCCGCGGTAA	Forward primer for V4 region of 16S rRNA gene[150]
806R	ATTAGAWACCCBDGTAGTCC	Reverse primer for V4 region of 16S rRNA gene[150]
M13_for	CACGACGTTGTAAAACGA	Forward mapping primer for the pJQ200SK vector
M13_rev	GGATAACAATTTCACACAGG	Reverse mapping primer for the pJQ200SK vector
M13_uni F	TGTAAAACGACGGCCAGT	Alternative forward mapping primer for the pJQ200SK vector
oxp0282	CCTACCTAGATCCTCGC	Forward mapping primer to confirm correct assembly of Golden Gate reactions in pOGG026[113]
oxp0283	AGCGTTCTGAACAAATCC	Reverse mapping primer to confirm correct assembly of Golden Gate reactions in pOGG026[113]
oxp0345	TTTTGGTCTCAGGCATGACTACAACCGTGCGC	Forward mapping primer for pJP2
oxp0675	TTTTGAAGACAAAAGCACGCCAAGATACGGC	Forward primer to amplify *fixC* in Rlv3841-based strains
oxp1483	TTTGGTCTCAGGAGTAGGGATAACAGGGTAATGACATCCCTCGATCCGACC	Reverse primer to amplify *fixC* in Rlv3841-based strains
oxp1857	TAGGGATAACAGGGTAATAAATTCGATCCAGGTCCCTACG	Forward primer to amplify *nifK* and flanking regions from RlvA34, with added I-*Sce*I homing endonuclease recognition site
oxp1858	ACGCCGCCTGTATTTAGAAG	Reverse primer to amplify *nifK* and flanking regions from RlvA34
oxp1859	TAGGGATAACAGGGTAATGTTTGAGACCGGCCCTTAC	Forward primer to amplify *nifK* and flanking regions from Rlp4292, with added I-*Sce*I homing endonuclease recognition site
oxp1860	GGTCGATATGCTGCGAGTTTC	Reverse primer to amplify *nifK* and flanking regions from Rlp4292
oxp1861	TTTGGATCCTATTCGTATGACCTCACTCGCT	Forward primer for inverse PCR from within *nifK* from RlvA34
oxp1862	TTTGGATCCCAAAGGCGCATGGTCGAGA	Reverse primer for inverse PCR from within *nifK* from RlvA34
oxp1863	TTTGAATTCTCTTACGACCTGACCCGCTA	Forward primer for inverse PCR from within *nifK* from Rlp4292

oxp1864	TTTGAATTCGGGAGCATGGTCGAGAACTTTT	Reverse primer for inverse PCR from within *nifK* from Rlp4292
oxp2310	TGATCTACGACGATGTGACTGG	Forward mapping primer to confirm in-frame deletion of *nifK* from RlvA34
oxp2311	TGTGTCCCATTATGATGTCGAT	Reverse mapping primer to confirm in-frame deletion of *nifK* from RlvA34
oxp2312	TTAAAGAGAAGTATCCGGTGCAGA	Forward mapping primer to confirm in-frame deletion of *nifK* from Rlp4292
oxp2313	TAAAAAGTTTCCGCTCGCCG	Reverse mapping primer to confirm in-frame deletion of *nifK* from Rlp4292
oxp2327	GATGATCTTCTCGCTGCCGA	Forward mapping primer for Tn*7* integrations into Rlv3841-based strains[147]
oxp2328	GCTCTGGCCAATGAGGTTCT	Reverse mapping primer for Tn*7* integrations into Rlv3841-based strains[147]
oxp2463	GGCCAAGGACACGAAAAGA	Forward mapping primer to confirm crossover of *nifK*::ΩSp^r with Rlp4292
oxp2464	TGGATTTCTTGACCGAGGTG	Reverse mapping primer to confirm crossover of *nifK*::ΩSp^r with Rlp4292
oxp2465	CTTTTCCCGCTAAGCAATGG	Forward mapping primer to confirm crossover of *nifK*::ΩSp^r with RlvA34
oxp2466	CCATGCCGATCTCCATCAA	Reverse mapping primer to confirm crossover of *nifK*::ΩSp^r with RlvA34
oxp2637	GCATGATGTCAGCCTTTCTCTC	Forward mapping primer to confirm crossover of *nifK*::ΩSp^r with RlvA34
oxp3060	CACAGCATAACTGGACTGATTTC	Reverse mapping primer for Tn*7* integrations using pOGG276-based plasmids
oxp3061	ATTAGCTTACGACGCTACACCC	Forward mapping primer for Tn*7* integrations using pOGG276-based plasmids
oxp3062	GAGCGCTTTTGAAGCTAATTCGA	Forward mapping primer to confirm correct assembly of Golden Gate reactions in the pOGG276 vector
oxp3063	TCACTTATCTGGTTGGCCTGC	Reverse mapping primer to confirm correct assembly of Golden Gate reactions in the pOGG276 vector
pIJ11268fw	CCATCTTTGCCCTACCGTAT	Forward mapping primer for pIJ11268-based plasmids
pIJ11268rv	AAACCGACGCCATCACCCAG	Reverse mapping primer for pIJ11268-based plasmids
pJET1.2 for	GACTCACTATAGGGAGAGCGGC	Forward mapping primer for the pJET1.2 vector
pJET1.2 rev	AAGAACATCGATTTTCCATGGCAG	Reverse mapping primer for the pJET1.2 vector
pOT forward_far	GACCTTTTGAATGACCTTTA	Universal mapping primer to amplify from within Ω Sp^r

Table 2.4 – Primers used in this thesis.

2.4 Bacterial growth

2.4.1 Growth conditions

E. coli strains were grown at 37°C, either in sterile LB broth shaken at 200 revolutions per minute (rpm), or on sterile solid LB media. *R. leguminosarum* strains were grown at 28°C, either in sterile TY[129] media shaken at 150 rpm, or on sterile solid TY agar. For long term storage strains were stocked in liquid LB or TY as appropriate with 15% glycerol, and flash frozen in liquid nitrogen before storing at -80°C.

2.4.2 Generation time

Generation times of strains were measured by growth curves to confirm that mutations, insertions, or plasmids did not negatively impact bacterial fitness. For each growth curve, an independent colony of each strain was grown on TY agar slopes with appropriate antibiotics at 28°C for three days. Under sterile conditions slopes were washed, diluted to 1 x 10^8 cells mL^{-1}, mixed 1:1 with liquid TY, then inoculated into 50 mL of liquid TY in a 250 mL conical flask to leave a final concentration of 1 x 10^6 cells mL^{-1}. Conical flasks were sealed with a sponge bung and placed in a 28°C shaker at 200 rpm. Optical density (OD_{600nm}) measurements were taken at four-hr time intervals to capture the exponential growth phase, where the OD_{600nm} is between 0.1 and 0.8, representing 1 x 10^8 and 8 x 10^8 cells mL^{-1}. A final measurement was taken at stationary phase (48 hours). At each time point, 1 mL of culture was collected, placed in a cuvette and the OD_{600nm} measured using a spectrophotometer. Generation time was calculated as the number of hours a population took to double in the exponential growth phase. Each growth curve was repeated three times independently and the mean generation time calculated for each strain.

2.5 Molecular techniques

2.5.1 DNA isolation

Genomic DNA was isolated from *R. leguminosarum* cultures using the DNeasy Blood and Tissue Kit (Qiagen). Plasmid DNA was extracted from *E. coli* using the GeneJET Plasmid Miniprep Kit (Thermo Fisher). To purify DNA following a restriction digest or PCR, the GeneJET PCR Purification Kit (Thermo Fisher) was used. DNA was isolated from electrophoresis gels using the GeneJET Gel Extraction Kit (Thermo Fisher). All kits were used according to the manufacturers' protocols.

2.5.2 PCR amplification

PCR primers were designed using Geneious version 10.0.9 and were synthesised by Eurofins Genomics. Primer annealing temperatures were determined by Geneious, and all reactions carried out in a Veriti® thermocycler (Applied Biosystems). Amplification PCR reactions were carried out using Phusion® High-fidelity DNA Polymerase (Thermo Fisher) using GC buffer and dimethyl sulfoxide (DMSO) according to the manufacturer's protocol. Colony PCR was used for mapping or screening reactions, and was carried out using either GoTaq® Green Master Mix (Promega) or OneTaq® Master Mix (New England Biolabs) according to the manufacturers' protocols. For colony PCR, colonies were selected using a sterile inoculation loop which was then mixed in 25 µL of sterile water, and 1 µL of this water was used as the DNA sample for the reaction.

2.5.3 Gel electrophoresis

Electrophoresis was used to separate PCR products or restriction digest fragments. Electrophoresis was carried out in 0.9% agarose (Sigma Aldrich) in TAE buffer pH 8.4 (400 mM Tris acetate, 1 mM ethylenediaminetetraacetic acid [EDTA]). Sybr® Safe at a

1:10000 ratio (Invitrogen) was added to the gel to stain the DNA. GeneRuler 1 kb or 1 kb Plus DNA ladders (Thermo Fisher) were used as marker ladders. Gels were run at 120 mV for 40 min. Stained gels were visualized using a GelDoc EZ System (Bio-Rad).

2.5.4 DNA sequencing

Sanger sequencing of plasmids and PCR fragments was carried out by Eurofins Genomics. Primers were designed using Geneious version 10.0.9 and premixed with samples according to the sequencing service's protocol.

2.5.5 Restriction digest and DNA ligation

Restriction digests were carried out using restriction endonucleases and their respective buffers (NEB, Roche, or Thermo Fisher) according to the manufacturers' protocols. DNA ligation was performed with T4 DNA ligase (Thermo Fisher) according to the manufacturer's protocol.

2.5.6 Transformation

Transformations were carried out using chemically competent *E. coli* α-Select Gold, DH5α, EC100D™ pir-116, or ST18 cells. Cells were thawed on ice in 1.5 mL Eppendorf tubes, then transformation DNA added in a 1:10 v/v ratio. Cells were incubated on ice for 30 min, heat-shocked for 45 s at 42°C, then incubated on ice for 2 min. 1 mL of SOC medium (Melford) was added to each Eppendorf, and Eppendorfs were placed in a 37°C shaking incubator at 200 rpm for 1 hour for recovery. The bacterial mixture was then plated on LB agar plates with appropriate antibiotics for selection, supplemented with X-gal where necessary for blue-white selection, and with ALA for ST18 cells, and colonies grown at 37°C overnight.

2.5.7 Golden Gate cloning

New marker plasmids were assembled using Golden Gate cloning[130]. This involved using genetic modules, which when digested by type IIs restriction enzymes, have sticky-ends which result in the DNA fragments assembling in the correct order in a vector[130,131]: the PU module (promoter with the ribosome binding site), SC module (open reading frame [ORF]), and the T module (terminator).

For each reaction 40 fmol of plasmid DNA for each component (PU module, SC module, T module, and vector) was combined in a PCR tube with 1.5 µL T4 DNA ligase buffer, 1.5 µL 2 mg mL^{-1} BSA, 1 µL BsaI to cut the common restriction sites of all modules, 1 µL T4 DNA ligase (2,000,000 units μL^{-1}) in a final volume of 15 µL. The reaction was carried out in a Veriti® thermocycler (Applied Biosystems): 25 cycles of 3 min at 37°C then 4 min at 16°C, followed by a final cycle of 5 min at 50°C then 5 min at 80°C. The reaction mix was transformed into competent cells and underwent blue-white selection using X-gal.

2.5.8 Conjugation from *E. coli* into *Rhizobium* strains

Plasmids were conjugated into *Rhizobium* strains by triparental mating. The recipient *Rhizobium* strains were grown on TY agar slopes for three days at 28°C, with selective antibiotics. *E. coli* containing the donor plasmids to be conjugated and the pRK2013 helper plasmid[148] were grown in 10 mL of liquid LB, with added selective antibiotics and ALA for ST18 cells, overnight in a 37°C shaker at 150 rpm. 100 µL of plasmid liquid culture was subcultured into 5 mL of liquid LB with the same selective antibiotics and

grown for 4 hours in a 37°C shaker at 150 rpm. *Rhizobium* slopes were washed with 5 mL liquid TY.

All *Rhizobium* and *E. coli* strains were then transferred to 2 mL Eppendorf tubes. Cultures were spun down at 6000 rpm for 5 min, then the supernatant was removed and re-suspended in liquid TY. This process was repeated three times to remove all traces of antibiotics. 2 mL Eppendorf tubes were set up for each conjugation, containing 400 µL of the donor *E. coli* plasmid strain, 400 µL of the recipient *Rhizobium* strain, and 200 µL of the pRK2013 helper plasmid strain. Control tubes were set up at the same volumes for each individual component. Tubes were spun down at 6000 rpm for 5 min, the supernatant discarded, re-suspended in 30 µL of liquid TY and plated on a sterilised nitrocellulose filter on a TY agar mating plate, with added ALA if ST18 cells were used. The nitrocellulose filters were left to dry for 10 min, then plates grown overnight at 28°C. The next day approximately half of the bacterial biomass was re-streaked from the nitrocellulose filter onto plates of TY agar with the appropriate antibiotics to select for the trans-conjugants. Strains were grown for three days at 28°C. Single colonies were selected and re-streaked on new selective plates, with this process repeated three times in total to purify the conjugated strain.

2.5.9 Mini-Tn*7* integration

Strains were marked chromosomally by mini-Tn*7* insertion[149,151], with the assistance of pTNS3[149]. pTNS3 contains the transposases *tnsABCD* which insert the marker gene into the region downstream of the *glmS* gene[149,152].

The method is similar to conjugation by triparental mating (**2.5.8**) except for the following changes. *E. coli* containing pTNS3 was additionally prepared, grown in 10 mL of liquid LB, with added ALA overnight in a 37°C shaker at 150 rpm. For subculturing, 500 µL of pTNS3 and donor plasmid liquid culture were used, to account for slower growth rates of the ST18 strains harbouring these plasmids. The mating plates contained ALA to account for the ALA auxotrophy of ST18 cells which harbour pTNS3. The conjugations contained 300 µL of the donor *E. coli* plasmid, 400 µL of the *Rhizobium* recipient, 300 µL of pRK2013, and 300 µL of pTNS3. The negative control consisted of 400 µL of the *Rhizobium* recipient, 300 µL of pRK2013, and 300 µL of pTNS3; and the donor control consisted of 300 µL of the donor *E. coli* plasmid. Instead of re-streaking from the nitrocellulose filter, the filter was washed in 1 mL of liquid TY, serially diluted, and 100 µL spread-plated from 10^0 to 10^{-2} dilutions on TY agar with appropriate antibiotics and grown for three days at 28°C. Individual colonies were selected for colony PCR to confirm the insertions.

2.6 Plant growth

2.6.1 Seed sterilisation and germination

Pea (*Pisum sativum* cv. Avola) or bean (*Phaseolus vulgaris* cv. Tendergreen*)* seeds were surface sterilised by washing in 95% ethanol for 30 s, rinsing once in sterile water, immersing in 2% NaClO for 5 min, and washing five times in sterile water. To germinate, seeds were transferred to 0.8% DWA plates under sterile conditions. Plates were wrapped in aluminium foil to exclude light and stored for five days at room temperature. Seeds with emergent radicles were selected for planting. Whether experiments used freshly sterilised or pre-germinated seeds is detailed for each experiment.

2.6.2 Rooting solutions

Plants were supplied with a sterilised rooting solution containing all essential nutrients except nitrogen. The solution for peas consisted of 4 mM Na_2HPO_4, 3.7 mM KH_2PO_4, 1 mM $CaCl_2.2H_2O$, 800 µM $MgSO_4.7H_2O$, 100 µM KCl, 35 µM H_3BO_3, 10 µM Fe EDTA, 9 µM $MnCl_2.4H_2O$, 0.8 µM $ZnCl_2$, 0.5 µM $Na_2MoO_4.2H_2O$, and 0.3 µM $CuSO_4.5H_2O$[153]. To account for the differing micronutrient requirement of beans, the rooting solution for beans consisted of 1 mM $CaCl_2.2H_2O$, 500 µM KH_2PO_4, 250 µM $MgSO_4.7H_2O$, 250 µM K_2SO_4, 10 µM ferric citrate, 1 µM $MnSO_4.H_2O$, 2 µM H_3BO_3, 0.5 µM $ZnSO_4.7H_2O$, 0.2 µM $CuSO_4.5H_2O$, 0.1 µM $CoSO_4.7H_2O$, and 0.1 µM $Na_2MoO_4.2H_2O$[154].

2.6.3 Planting and inoculation

Plants were grown in Azlon pots, the size of the pot and the substrate used is detailed individually for each experiment. Pots containing either fine (1-3 mm) or medium (2-5 mm) washed and drained vermiculite had 400 mL of the appropriate nutrient solution added per litre of substrate before autoclaving. Pots containing a mixture of 1:1 fine vermiculite and silver sand were autoclaved without nutrient solution; then sterile nutrient solution was added at planting. 2.67x concentrated nutrient solution was used to adjust for the use of sand as a substrate, with 150 mL of the appropriate solution used per litre of substrate.

Seeds were planted and inoculated under sterile conditions. The number of seeds placed per pot, whether these seeds were pre-germinated or not, and the number of rhizobium cells used to inoculate seeds is detailed individually for each experiment. Pots were covered in cling-film to maintain sterile conditions, which was slit after two to five days

using a sterile scalpel to allow for growth. When multiple seeds were planted per pot, seeds were generally thinned after seven days to one per pot using sterile tweezers.

2.6.4 Growth conditions

Plants were grown under a 16-hour-light/8-hour-dark diurnal regime at 21°C. Peas were watered with sterile water after seven days when necessary. When peas were grown to 56 days post inoculation (dpi), they were watered with 150 mL sterile 2.67x N-free rooting solution at 28 dpi[153]. Beans were watered with 150 mL sterile N-free rooting solution per week[154], and with extra sterile water when necessary. Plants were harvested between 12 and 56 dpi depending on the experiment. Upon harvest, the substrate was gently tipped out of the pot, then roots gently shaken. If plants were not to be used for acetylene reductions, the roots were carefully washed with water.

2.7 Post-harvest procedures

2.7.1 Acetylene reductions

Acetylene reductions were used to measure nitrogen fixation rates of bacteroids in nodules. The nitrogenase enzyme reduces atmospheric dinitrogen (N_2) to ammonia (NH_3), but can also reduce acetylene (C_2H_2) to ethylene (C_2H_4) due to the similarity between the triple bonds in nitrogen and acetylene[155]. Nitrogenase has greater affinity for acetylene reduction over nitrogen reduction[156], and therefore the percentage of acetylene converted to ethylene can be used to measure nitrogenase activity.

After harvest, plants inoculated with a single strain were transferred to 250 mL Duran bottles (total volume 320 mL) with airtight neoprene lids, along with some wet tissue paper to prevent the samples drying out. Using a syringe, 8 mL of air was removed from

each bottle and replaced by 6.4 mL acetylene to a final concentration of 2% acetylene. This low concentration of acetylene was used to minimize the risk of acetylene-induced decline of nitrogenase activity[157]. After one hour incubation at room temperature, three 1 mL gas samples were taken from the jar using syringes, the syringes were stored in a rubber bung to prevent gas loss, and then samples analysed for acetylene and ethylene using a gas chromatogram (Clarus 480, PerkinElmer). The percentage of acetylene reduced to ethylene was used to calculate the rate of nitrogenase activity $plant^{-1}$ $hour^{-1}$, and averaged across the three technical replicates to give a single data point per plant.

For acetylene reductions of plants co-inoculated with two strains, roots were carefully laid out on 120 x 120 mm^2 plates, placed on a blue-light transilluminator (VWR), and viewed through an orange ultraviolet (UV) filter. All GFP nodules were removed, leaving just mCherry nodules on the plant. Plants were kept moist during this process, and the order of plants to have their nodules removed was randomised. Plants then underwent acetylene reduction as above.

2.7.2 GusA and CelB staining

GusA and CelB chromogenic staining was used to distinguish between nodules containing strains marked with either *gusA* or *celB* genes[135,158]. Roots were cut off from the plant at the substrate level, gently washed with water, and placed in Falcon tubes covered with aluminium foil containing 40 mL phosphate buffer (7 g L^{-1} NaH_2PO_4, 7.2 g L^{-1} Na_2HPO_4, 1 mM EDTA [pH 8], 1% Sarkosyl, 1 mL L^{-1} Triton).

For experiments where a *gusA*-marked strain was co-inoculated with an unmarked strain, X-glcA (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) was added to a final

concentration of 0.2 mg mL^{-1} and roots incubated overnight at 28°C. GusA converts X-glcA into a blue precipitate, staining these nodules blue[159]. Roots were then washed and carefully laid out on 120 x 120 mm^2 plates for photography.

For experiments where a *gusA*-marked strain was co-inoculated with a *celB*-marked strain, Magenta-glcA (5-bromo-6-chloro-3-indolyl-β-D-glucuronide) was added to a final concentration of 0.2 mg mL^{-1} and roots incubated overnight at 28°C. GusA also converts Magenta-glcA into a magenta precipitate, staining these nodules magenta[158]. Roots were then washed, transferred to fresh preheated phosphate buffer, and incubated in a water bath for 2 hours at 70°C to destroy endogenous β-galactosidase activity. X-gal was then added to a final concentration of 0.25 mg mL^{-1} and roots incubated overnight at 37°C. CelB, a thermostable β-glucosidase with high β-galactosidase activity, converts X-gal into a blue precipitate, staining these nodules blue[158]. Roots were then washed and carefully laid out on 120 x 120 mm^2 plates for photography.

2.7.3 Root system photography

To image root systems, roots were washed and carefully laid out on 120 x 120 mm^2 plates, so all nodules were visible. To image root systems on a white background, roots were photographed using a Nikon D300 or D700 camera with no filter, no flash, and exposure time as needed. To image fluorescently marked nodules, roots were placed on a blue-light transilluminator (VWR) and photographed using a Nikon D700 camera (55 mm Micro-Nikkor f/2.8 lens, orange filter [Hoya Orange (G)], f/22 aperture, and 10 s exposure).

2.7.4 Microscopy

Microscopy images of nodules were taken using a dissecting microscope (Leica M165 FC) with a Leica DFC310 FX digital camera using LAS v4.5 software. Images were taken using brightfield imaging, a GFP3 filter (475-495 nm excitation wavelength, 505-540 nm emission wavelength), or an mCherry filter (540-580 nm excitation wavelength, 593-668 nm emission wavelength), with exposure times as needed.

2.7.5 NightOWL imaging

For detecting light emission from strains containing luminescent biosensors, roots were gently washed and carefully laid out on 120 × 120 mm^2 plates so all nodules were visible. Plates were imaged with a NightOwl II camera (Berthold Technologies) at 60 s exposure using inbuilt IndiGO software v2.0.5.0. Luminescence was detected, and only nodules emitting over 40 counts per second (cps) of luminescence (to avoid background noise) were counted automatically using the IndiGO software, with manual curation to ensure the luminescent areas aligned with the root system.

2.7.6 Fluorescent nodule identification

Nodules were selected for viability and flow cytometry experiments based on their fluorescence. Single-strain nodules were selected using images taken with a blue-light transilluminator (VWR) and an orange filter (Hoya Orange [G]) (**2.7.3**). Mixed nodules were selected using fluorescence microscopy (**2.7.4**). The number of nodules selected is detailed for each experiment.

2.7.7 Nodule surface sterilisation and extraction

After selection, nodules were surface sterilised by immersing in 2% NaClO for 60 s, then washed five times in sterile H_2O. Rhizobia were extracted by placing nodules in a 1.5 mL Eppendorf tube and crushing with a pestle in 1 mL of filter-sterilised bacteroid isolation buffer (8.2 mM K_2HPO_4, 1.98 mM KH_2PO_4, 300 mM sucrose, and 2 mM $MgCl_2$).

2.7.8 Nodule extract patch plating

Patch plating was conducted after removing all nodules from a plant, surface sterilisation, and crushing to extract rhizobia. Extracts were serially diluted in bacteroid isolation buffer, 100 µL of each dilution from 10^{-3} to 10^{-5} was spread-plated on TY agar with nystatin and appropriate antibiotics and grown for 3 days at 28°C. 100 colonies were then patched using a sterile toothpick onto both non-selective TY agar and selective TY agar, grown three days at 28°C, and colony growth counted after three days.

2.7.9 Inoculation and nodule extract drop plating

Drop plating was conducted to confirm the number of rhizobia used to inoculate plants or count the number of colony forming units (CFUs) in nodule extracts. Extracts were serially diluted in water (inoculations) or bacteroid isolation buffer (nodule extracts), and five droplets (for five technical replicates) of 25 µL for a range of appropriate dilutions were spotted onto 120 x 120 mm^2 plates containing nystatin and appropriate antibiotics. Droplets were left to dry, then plates were sealed and grown at 28°C for two days. The number of colonies from inoculations or single-strain nodule extracts were counted manually. The number of GFP-marked and mCherry-marked colonies from mixed nodule extracts were counted by imaging colonies using a dissecting microscope as in **2.7.4,** and

images used for manual counting. The number of colonies was averaged across the five technical repeats for each sample.

2.7.10 Nodule extract flow cytometry

Nodule extracts for flow cytometry were first processed to purify the samples. All reagents used in flow cytometry were filter-sterilised using a 0.22 μm filter on the day of use to prevent precipitates contaminating the results. Extracts were centrifuged at 1000 rpm for five min to pellet the plant debris. The supernatant was extracted, centrifuged at 6000 rpm for five min to pellet the rhizobia, then resuspended in 1 mL 0.9% NaCl, and this process was repeated for a total of three times. Extracts from experiments where multiple (ten) nodules were selected from the plant were then diluted 10^{-1} in flow cytometry grade phosphate-buffered saline (PBS) (Thermo Fisher), whilst extracts from single nodules were not diluted.

Free-living rhizobia were also prepared as unmarked and marked controls for compensation. Compensation adjusts the results to account for spill-over of one fluorescent protein into other detection channels[160]. Unmarked, GFP-marked, and mCherry-marked Fix^+ rhizobial strains (plus BFP-marked when needed) were grown on TY agar slopes with appropriate antibiotics for three days at 28°C. Slopes were washed with 5 mL 0.9% NaCl, then 1 mL centrifuged at 6000 rpm for five min and resuspended in 1 mL 0.9% NaCl a total of three times. Samples were diluted 10^{-2} in flow cytometry grade PBS (Thermo Fisher).

Flow cytometry was conducted using an Amnis® CellStream® flow cytometer (Luminex). 100 μL each of the unmarked and marked controls for compensation were measured in

all channels but SSC as detailed in **Table 2.5**, and 5000 events were recorded per control. Nodule samples were analysed in 96-well plates. 80 µL of sample was added to odd-numbered wells, and 35 µL of 0.9% NaCl added to even-numbered wells to wash the flow cytometer between samples. NaCl was used to break down exopolysaccharides (EPS) which cause rhizobia to aggregate[161], as trials showed this was necessary to prevent cross-contamination between samples. Each sample was repeated in three wells, to give three technical repeats. 5000 events were recorded per sample in slow/high sensitivity mode to maximise the resolution of the small rhizobial cells[162] using the appropriate channels for the sample (**Table 2.5**). The number of events was used as an end point due to the low cell density within some samples, rather than flow time as in previous studies[63,163].

Channel name	Laser	Laser power (%)	Detector	Cell type
Forward scatter (FSC)	450 nm LED	30	456/51 mm	All
Side scatter (SSC)	785 nm	100	733/56 mm	All
Blue	405 nm	100	456/51 nm	BFP and CFP
Green/yellow	488 nm	100	528/46 nm	GFP and YFP
Red	532 nm	100	611/31 nm	mCherry

Table 2.5 – Channels used for flow cytometry.

2.8 Computational methods

2.8.1 Cloning

Geneious version 10.0.9 was used to test *in silico* cloning reactions, and to align sequencing results against references to confirm *in vitro* cloning results.

2.8.2 Nodule counting and area measurement

Nodules were counted from root system photographs (**2.7.3**) to obtain the total number of nodules per plant, or the number of nodules of each type when labelled using *gusA* and *celB* markers or fluorescent proteins. Nodules were counted manually using the Cell Counter plugin from the Fiji distribution[164] of ImageJ version 1.53a or 1.53c[165]. Nodules were counted three times independently, and the mean of the three counts calculated. Fiji was also used for measuring nodule areas. The scale of the image was set using 10 mm of the scale bar on the image, then nodules outlined using the polygon selections tool before measuring the area inside each polygon. Nodule size per strain per plant was estimated by measuring the area of the largest (most developed) nodules by area, to account for variation in size due to developmental stage. The largest twenty nodules by area per strain per plant as judged by the experimenter were measured (or as close to twenty as possible if fewer than twenty were present). Then in most cases, the results for the ten largest nodules were kept, though for the partner choice experiment (**5.4.4**) only the results for the five largest nodules were kept for the 1:10 and 10:1 inoculation ratios, as these plants did not all have ten nodules of each strain.

2.8.3 Flow cytometry data analysis

Flow cytometry data was analysed using CellStream® Analysis software version 1.3.382 (Luminex). Before analysis, a compensation matrix was calculated using the compensation samples, and this compensation matrix was applied across all samples to adjust for spill over into other detection channels. Then the data were analysed. First a gate was applied to identify GFP- and mCherry-marked cells. GFP-marked cells were defined as those with an intensity over 8000 arbitrary units (AU) in the green/yellow channel (**Figure 2.1*A***), and mCherry cells as those with an intensity over 4000 AU in the

red channel (**Figure 2.1*B***). Cells expressing both GFP and mCherry were excluded from further analysis as these were likely to be doublets (where two cells have passed the detector at the same time[166]). The fluorescently labelled cells were then separated from small background particles by selecting cells with both FSC (size) and SSC (granularity) intensities over 300 AU (**Figure 2.1*C***). The cells were separated into bacteria and bacteroids using FSC as this gives information on cell size[167]. Cells under 5000 AU were defined as bacteria, and cells over 5000 AU as bacteroids, as this allowed good distinction

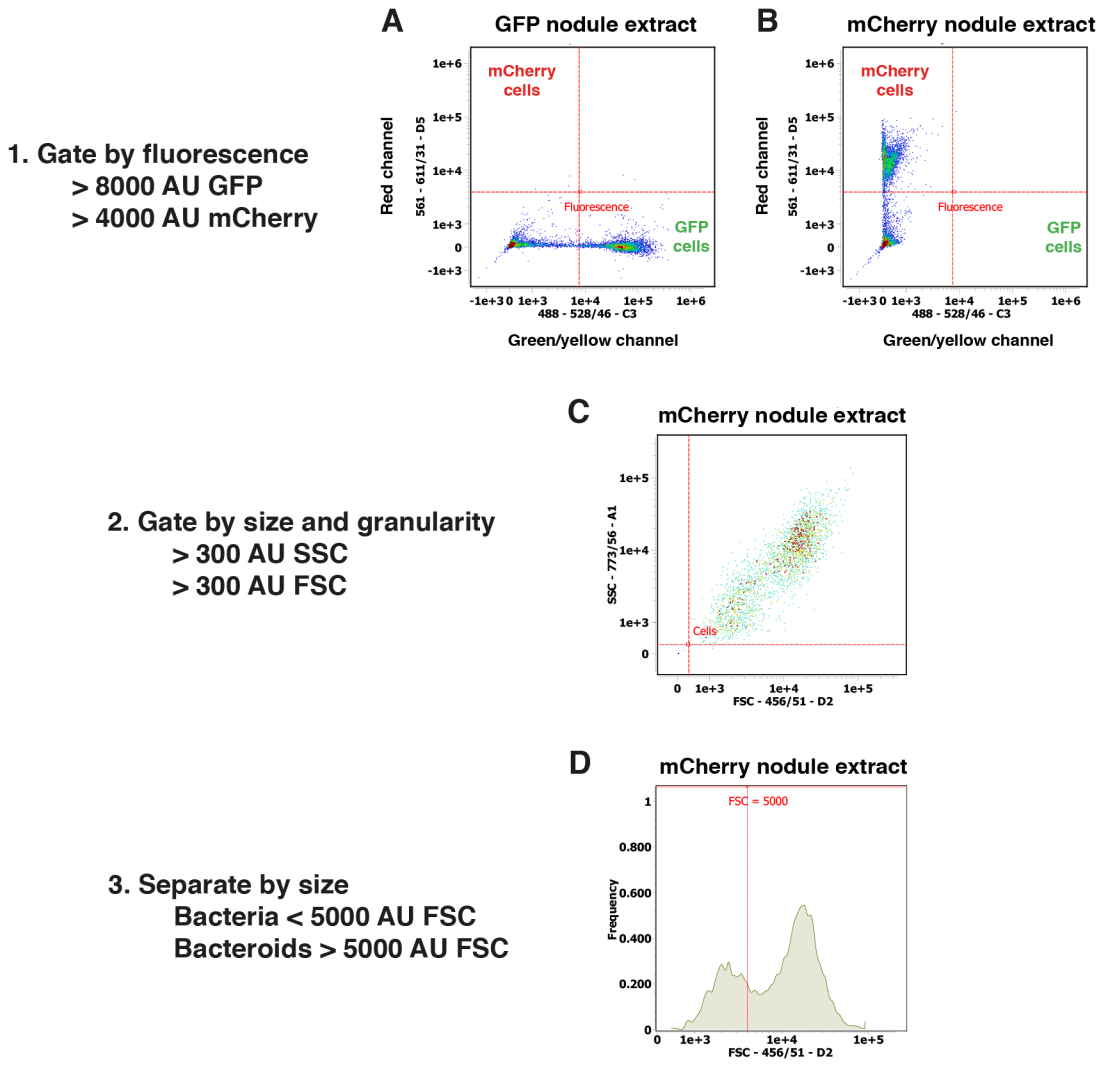


Figure 2.1 – Flow cytometry gating procedure. (A) Heatmap showing the emission profile of a GFP nodule extract. (B) Heatmap showing the emission profile of a mCherry nodule extract. (C) Heatmap showing the FSC and SSC profile of mCherry-marked cells identified from a mCherry nodule extract. (D)

Histogram showing FSC of mCherry-marked cells identified from a mCherry nodule extract. (A-D) Graphs are representative of flow cytometry samples for Fix^+ nodules.

between the two cell types using FSC histograms (**Figure 2.1*D***). Finally, the number of mCherry- or GFP-marked bacteria and bacteroids were counted, and the volume of the sample was used to calculate the events mL^{-1} and events $nodule^{-1}$. This value was averaged across the three technical repeats for each sample.

2.8.4 Statistics

Analyses were conducted using R version 3.6.3[168]. Data were processed with the assistance of the packages dplyr version 1.0.2[169] and reshape version 0.8.8[170]. Mixed-effects models were fitted using lmer from the package lme4 version 1.1-25[171] and *p*-values were calculated by the package lmerTest version 3.1-3[172]. Group means and 95% Cis were calculated from models with the assistance of the package emmeans version 1.5.4[173]. The details of analyses conducted are included for each individual experiment. Graphs were produced using the package ggplot2 version 3.3.2[174], with colour palettes from the package RcolorBrewer version 1.1-2[175], and the package scales version 1.1.1[176] used to customise graph axes. Figures were compiled using Adobe Illustrator version 25.0.1.

Chapter 3 Conditional sanctioning of an intermediate fixer

3.1 Abstract

Plants host rhizobia in specialised root nodules and provide the rhizobia with carbon and other nutrients in return for nitrogen. But plants face an investment dilemma, as they usually house multiple strains of rhizobia that can vary in their nitrogen fixation ability. A poorly fixing strain could cheat the plant by leaving the costly process of nitrogen fixation to other strains, while gaining higher fitness through investing more in reproduction. This potential conflict between partners could cause the breakdown of the symbiosis over evolutionary time.

While plants are known to sanction strains that do not fix nitrogen, non-fixers are rare in field settings, while intermediate fixers are common. Here we inoculated pea (*Pisum sativum)* plants with different combinations of otherwise isogenic fixing, intermediate-fixing, and non-fixing derivatives of the standard laboratory rhizobial strain *Rhizobium leguminosarum* bv. *Viciae* 3841. We tested whether the host response to the intermediate-fixing strain was dependent on which other strains were available to the plant.

Intermediate fixers were only tolerated when a better strain was not available. Nodules containing the intermediate fixer were large and healthy when the only alternative was a non-fixer. When a more effective strain was available, plants downregulated carbon transport to the intermediate fixer, and nodules containing the intermediate fixer were smaller, with reduced nitrogen fixation. At a later stage of development, such nodules also contained fewer culturable rhizobia. This indicates that legumes can make conditional decisions, giving them remarkable control over their symbiotic partners.

3.2 Overview

Some data presented in this chapter have been published in:

Conditional sanctioning in a legume–*Rhizobium* mutualism

Westhoek, A., Clark, L. J., Culbert, M., Dalchau, N., Griffiths, M., Jorrin, B., Karunakaran, R., Ledermann, R., Tkacz, A., Webb, I., James, E. K., Poole, P. S. & Turnbull, L. A.

Proceedings of the National Academy of Sciences of the United States of America **118,** e2025760118 (2021)[108]

The published manuscript can be found in **Appendix 2**. The data that support the findings of this manuscript are available in the Oxford University Research Archive (DOI: 10.5287/bodleian:6qmxaR7db).

The data in this chapter which were included in the above publication are detailed in **Table 3.1**.

The data which support this chapter are available in the Oxford University Research Archive (DOI: 10.5287/bodleian:zBpJPja9E).

3.3 Introduction

Legume plants can obtain nitrogen through a symbiosis with nitrogen-fixing bacteria, known as rhizobia. The plant hosts rhizobia in specialised root nodules and provides the rhizobia with carbon and other nutrients in return for nitrogen[14]. But plants face an investment dilemma, as they usually house multiple strains of rhizobia[78–81] that can vary in their nitrogen fixation ability and therefore in how much nitrogen is provided to the host[27,75,76,177]. Potentially, rhizobia could be selected to cheat by leaving the costly process of nitrogen fixation[82] to other strains, while gaining higher fitness through investing more in reproduction[83,84]. This potential conflict between partners could cause the breakdown of the symbiosis over evolutionary time[85,86].

Experimental evidence shows that nodules containing strains that provide no nitrogen show restricted development, with smaller nodules and reduced fitness of the rhizobia inside[90,93,105–107]. This has been termed sanctioning, and the ability to sanction could have a two-fold effect on this symbiosis. Firstly, from an individual plant perspective, this would prevent the plant wasting resources on strains that provide no nitrogen[84]. Secondly, from an evolutionary perspective, the resulting reduced rhizobial fitness may prevent the spread of ineffective strains through the population which could otherwise be invaded by non-fixers.

However, experimental evidence does not address how host plants would respond to rhizobial strains which have a range of nitrogen-fixing abilities. This is crucial, as in the field non-fixing strains are probably rare, while poor and intermediate fixers are common[27–29]. If the host plant can assess the fixation ability of its partners, this could allow the host to maximise its own growth rate by conditionally sanctioning strains:

sanctioning a strain only when a better strain is available. However, experimental results obtained so far cannot distinguish whether plants conditionally sanction their partners, or if plants allocate resources to strains in proportion to their fixation ability.

Proportional sanctioning of nodules with a range of fixation rates was demonstrated by Kiers *et al.* (2006) for a host plant with just one strain available. Here argon gas was used to replace nitrogen gas at different proportions, reducing nitrogen availability to nodules and forcing a range of decreased nitrogen fixation rates. The resultant nodule size and rhizobial fitness was reduced in proportion to the nitrogen fixation rate of the nodule[106]. However, this study did not investigate what happens to an intermediate-fixing nodule when it is the best available option for the plant. Therefore, it is unknown if the same proportional response would be seen when the intermediate-fixing nodule is the best available option for the plant, or whether the host would increase investment in this nodule.

Studies comparing strains with different fixation rates on the same plant have been carried out using naturally-occurring strains of rhizobia which were collected from the field[77,88,95]. Here the results are less definitive, in one study the more effective strain did not have a fitness advantage[77], but in others the more effective strain had larger nodules[88] or a fitness advantage[95]. However, these strains may have differed in traits other than their fixation ability, such as nodulation competitiveness[100] and metabolic demands[63], so any observed host response could be due to a combination of these traits. Additionally, these studies could not assess conditional sanctioning, as they did not compare the host response to an intermediate strain when it was the best option, and the worst option, for the host plant.

To determine the host response to differences in nitrogen fixation only, fixation ability could be altered by modifying key nitrogen fixation genes and then these otherwise isogenic strains could be compared. However, so far these studies have only investigated non-fixers, not intermediate fixers[105,107]. Therefore, experiments comparing a genetically altered intermediate fixer with otherwise isogenic fixing and non-fixing strains would be key to determine whether host plants can conditionally sanction their rhizobial partners.

We tested conditional sanctioning using pea (*Pisum sativum)* plants, inoculated with different combinations of otherwise isogenic fixing (Fix^{+}), intermediate-fixing (Fix^{int}), and non-fixing (Fix^{-}) derivatives of the standard laboratory rhizobial strain *Rhizobium leguminosarum* bv. *Viciae* 3841 (Rlv3841). The differing levels of nitrogen fixation were caused by the insertion of a Ω spectinomycin resistance (Sp^{r}) cassette, in the region between *fixX* and *nifA* for Fix^{int} [108], or in the *nifH* gene for Fix^{-}[105].

When multiple strains were inoculated on the same plant, strains were distinguished using chromogenic or fluorescent makers. GusA and CelB chromogenic staining[135] was used to distinguish between strains when counting nodules or measuring nodule size. This staining process is fatal for rhizobia, so for experiments requiring identification of nodules containing live rhizobia, strains were chromosomally marked with GFP or mCherry fluorescent proteins[149,151]. Luminescent biosensor plasmids were used to visualise the expression of genes involved in nitrogen fixation and carbon transport[108,140,143]. Each biosensor contains *luxCDABE* genes, which cause rhizobia to luminesce, under the control of a specific promoter so that they are co-expressed with other genes at key stages of nodule development. The P*nifH* biosensor luminesces when

nitrogenase genes are expressed during nodule development. The C4-dicarboxylates and sucrose biosensors are indicators of carbon supply to strains, as they luminesce when the *dctA* transporter (transporting C4-dicarboxylates) and the sucrose transporter are transcribed[143]. The constitutive P*neo* biosensor was used as a positive control, and an indicator of background transcription activity[140]. We tested these markers to ensure they had no deleterious effect on the rhizobia which could confound results when competing differentially marked strains against each other.

We then tested whether the host plant can conditionally sanction the Fix^{int} strain: specifically, if Fix^{int} is only sanctioned when it is competing against a better strain. Firstly, as sanctioned strains are often observed to have smaller nodules[88,90,93,105,106], we determined if the size of Fix^{int} nodules is conditional on the identity of the competing strain. Second, in an attempt to elucidate the mechanism of sanctioning, we assessed if the carbon supply to Fix^{int} is conditional on the identity of the competing strain. Third, we assessed if the nitrogen fixation ability of Fix^{int} is conditional on the identity of the competing strain. Finally, we counted the number of colony forming units (CFUs) in Fix^{int} nodules to determine if conditional sanctioning impacts rhizobial fitness, and therefore could be a mechanism by which hosts select for more effective partner strains.

The experiments and analyses described in this chapter are all my own work. However, some data presented in this chapter are the work of contributing researchers (Annet Westhoek, Andrzej Tkacz, and Megan Griffiths; all University of Oxford). Andrzej Tkacz conducted whole genome sequencing and mapping of the intermediate fixer. Annet Westhoek measured the nodule sizes in the single and triple inoculation nodule size experiments. Data from my single and double inoculation nodule size experiments was

combined with further experimental replicates by Annet Westhoek and Megan Griffiths to obtain adequate sample sizes for publication. The data presented here include the data from these researchers and is clearly labelled, as an adequate sample size was required to draw conclusions. **Table 3.1** details where other researchers contributed to the data in this chapter, and how this data was combined.

Reference	Data type	Published state of data	Contributors
3.4.1.1	Genome mapping of Fix^{int}	As described in this thesis	Whole genome sequencing and mapping conducted by Andrzej Tkacz
3.4.2.1	Nitrogen fixation rates and nodule sizes in single inoculations	As described in this thesis	NA
3.4.2.1	Nodule sizes in single inoculations	As described in this thesis	Nodule sizes were measured by Annet Westhoek. Data were combined with one experimental repeat by Annet Westhoek and Megan Griffiths
3.4.3.1	Nodule sizes and nodule counts in double inoculations	As described in this thesis	Data were combined with two experimental repeats by Annet Westhoek and Megan Griffiths
3.4.3.2	Nodule sizes in triple inoculations	As described in this thesis	Nodule sizes were measured by Annet Westhoek
3.4.6	Carbon transport data from luminescent biosensors in double inoculations	Data from the P*neo* biosensor were excluded for publication	NA
3.4.7	Nitrogen fixation rates in double inoculations	As described in this thesis	NA
3.4.8	Fitness in double inoculations	As described in this thesis	NA

Table 3.1 – Data from this thesis which were published in Westhoek *et al*. (2021)[108], and which researchers contributed to this data.

3.4 Results

3.4.1 Confirmation of the Fix^{int} genotype

3.4.1.1 PCR of Fix^{int}

The Fix^{int} strain used in this thesis, OPS1474, was derived from strain LMB827 which was originally intended to be a *fixC*::ΩSp^{r} mutant[178]. This strain was isolated by the insertion of the plasmid pLMB878 (**Figure 3.1*A***) into Rlv3841, resulting in a strain with unexpected intermediate fixation rates[178,179] and nodule sizes[179]. Whole genome sequencing was conducted to determine the cause of the intermediate fixation rates[108], which demonstrated that LMB827 was actually a mixture of two strains. One strain, OPS1475 (**Figure 3.1*C***), was *fixC*::ΩSp^{r} as intended, as a double-crossover event replaced *fixC* with *fixC*::ΩSp^{r} (**Figure 3.1*B***). In the second strain, OPS1474 (**Figure 3.1*E***), the ΩSp^{r} cassette was not inserted in *fixC*, but instead was inserted between *nifA* and *fixX*. This prevented readthrough of the operon from the NifA-dependent promoter upstream of *fixA* (**Figure 3.1*D***)[108]. We predicted that this would disrupt transcription of genes downstream of *fixB* (i.e. *fixC*, *fixX* and *nifA*) and prevent full auto-upregulation of the operon by NifA under nitrogen-fixing conditions, hence the intermediate fixation phenotype.

To confirm that LMB827 contained a combination of strains with *fixC*::ΩSp^{r} and unmutated *fixC*, and to purify the two strains, colony PCR was conducted on individual colonies of LMB827. Primers 515F and 806R amplify 16S rRNA[150] and were used as a positive control. Primers oxp0675 and oxp1483 amplify a region within *fixC* and were used to test for the presence of *fixC*. All colonies (n = 20) showed the expected 292 bp band for the positive control, and 15 colonies showed the expected 518 bp band for *fixC*.

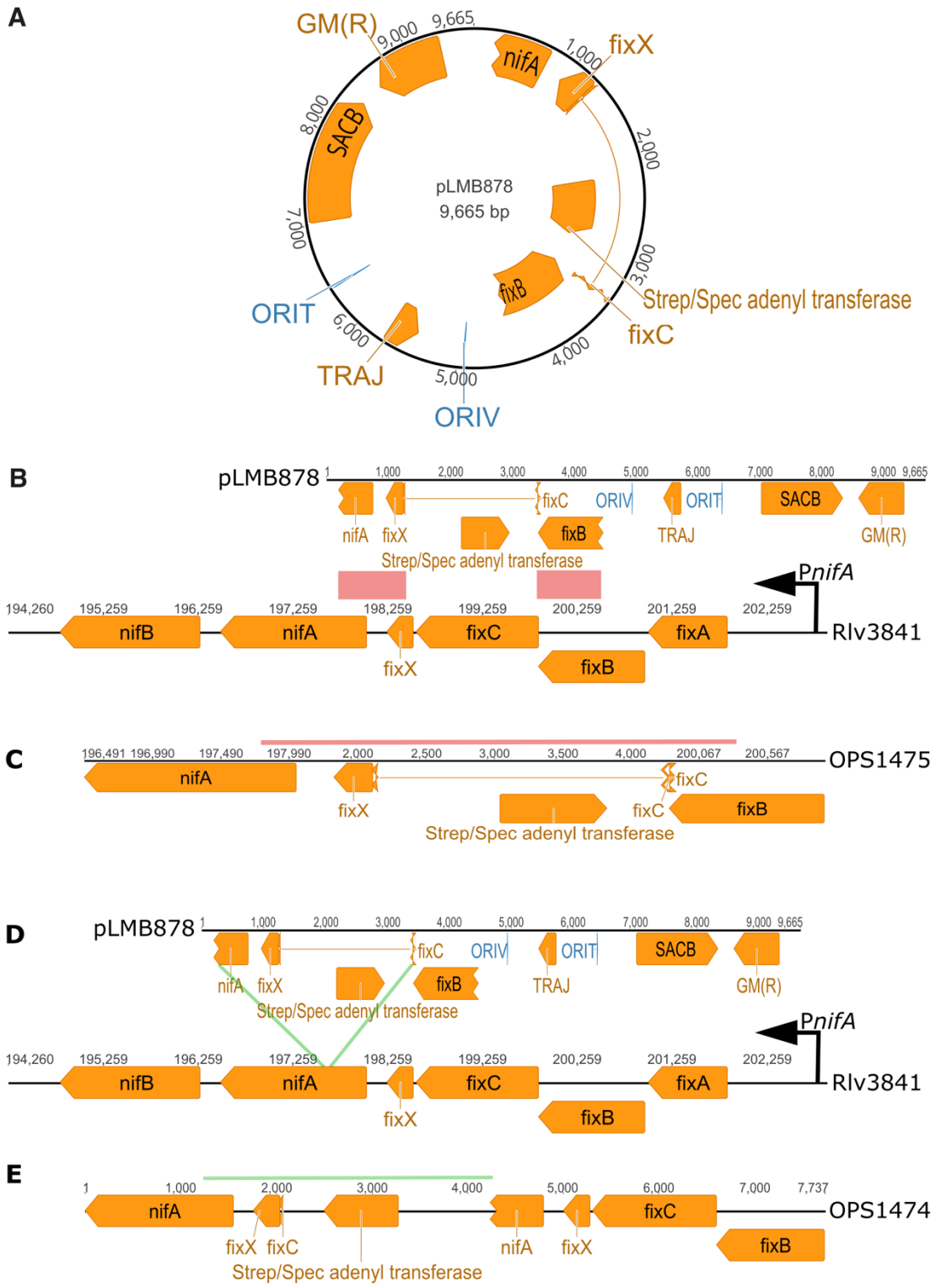


Figure 3.1 – OPS1474 and OPS1475 mutagenesis. (A) Plasmid pLMB878 was used to insert a ΩSp^r cassette. (B) The ΩSp^r cassette replaced *fixC* in Rlv3841 in a double-crossover event, indicated by the red area, to give (C) OPS1475, where the insert is indicated by the red line. (D) The ΩSp^r cassette was inserted in Rlv3841 in between *nifA* and *fixX* in the location indicated by the green lines to give (E) OPS1474, where the insert is indicated by the green line. Instead of disrupting *fixC,* read through from the NifA-dependent promoter (P*nifA*) upstream of *fixA* is prevented. (B, D) Direction of the P*nifA* promoter is indicated by the arrow.

This demonstrated that LMB827 contained a combination of strains, with 25% of colonies as *fixC*::ΩSp^r.

One colony of each type was stocked, OPS1474 with *fixC* and OPS1475 with *fixC*::ΩSp^r. A further ten colonies of OPS1474 and OPS1475 were tested to confirm their identity. All ten colonies of OPS1474 had the expected 292 bp band for the positive control and the 518 bp band for *fixC.* Nine colonies of OPS1475 had the 292 bp band for the positive control, and of these nine, none had the 518 bp band for *fixC*. This demonstrated that OPS1475 is a *fixC*::ΩSp^r mutant (**Figure 3.1*C***), and that OPS1474 has an undisrupted *fixC* and is therefore the strain with ΩSp^r between *nifA* and *fixX* (**Figure 3.1*E***).

3.4.2 Assessment of the Fix^{int} phenotype

3.4.2.1 Nitrogen fixation and nodule size in single inoculations

To determine the effect of the ΩSp^r cassette insertion on the nitrogen fixation rates of OPS1474 and OPS1475, the rates of acetylene reduction were measured.

Plants were inoculated with single inoculations of OPS1474, OPS1475, *gusA*-marked Fix$^+$ (Rlv3841 *gusA*), and *celB*-marked Fix$^-$ (OPS0366). One pre-germinated pea seed was potted per 500 mL pot containing a 1:1 mixture of silver sand and fine vermiculite and inoculated with 1 x 10^2 cells per pot. Four replicate plants per treatment and four water controls were grown for 27 days in a fully randomised design. Rates of acetylene reduction were measured, the number of nodules counted, and the shoot dry biomasses measured. Acetylene reduction rates and shoot dry biomass were analysed as a function

of strain using a linear model. Nodule number was analysed as a function of strain using a generalised linear model with a quasipoisson error distribution.

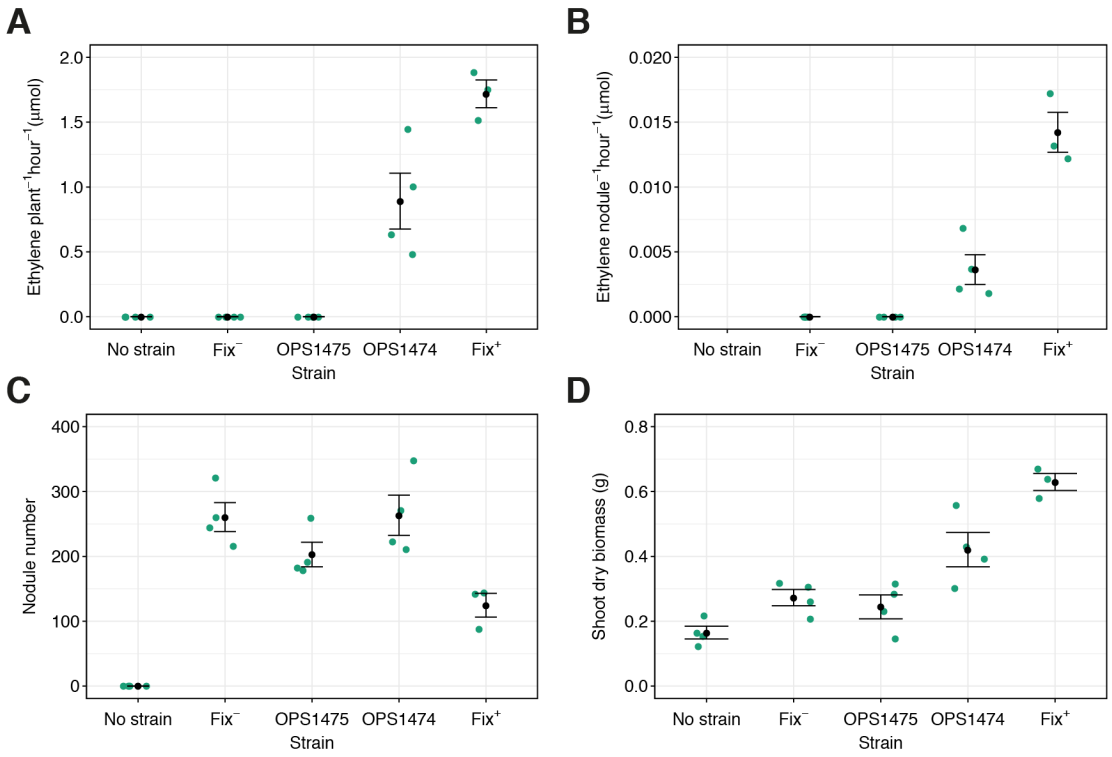


Figure 3.2 – The nitrogen-fixing ability of Fix$^+$ and its derived mutants. (A) OPS1475 does not fix nitrogen, while OPS1474 fixes at 52% of the Fix$^+$ rate when expressed per plant. (B) OPS1475 does not fix nitrogen, while OPS1474 fixes at 26% of the Fix$^+$ rate when expressed per nodule. (C) OPS1475 and OPS1474 both form as many nodules as Fix$^-$, which is more than Fix$^+$. (D) OPS1475 has a similar shoot dry biomass to Fix$^-$, while OPS1474 has an intermediate shoot dry biomass. (A, B) Points represent an average of three technical replicates per independent replicate plant, (C, D) points represent each independent replicate plant. (A-D) Black points indicate the overall mean with one standard error. Plants were grown for 27 days, n = 3-4.

In single inoculations, acetylene reductions revealed that OPS1475 did not fix nitrogen, and OPS1474 fixed nitrogen at reduced rates compared to Fix$^+$. Per plant, OPS1475 did not fix any nitrogen, while OPS1474 fixed less than Fix$^+$ (a difference of 0.83 µmol ethylene plant^{-1} hour^{-1}, 95% CI [0.48, 1.17], **Figure 3.2*A***), a reduction of 48%. When expressed per nodule, again OPS1475 did not fix nitrogen, while OPS1474 fixed less than Fix$^+$ (a difference of 0.011 µmol ethylene nodule^{-1} hour^{-1}, 95% CI [0.008, 0.013], **Figure 3.2*B***), a reduction of 74%.

Plants inoculated with OPS1475 and OPS1474 formed on average 203 and 263 nodules respectively, similar to plants inoculated with Fix$^-$ (mean 261; ns difference of 58 to OPS1475, risk ratio [RR] 1.28, 95% CI [1.00, 1.65]; ns difference of 2 to OPS1474, RR 0.99, CI [0.76, 1.28]), and more than plants inoculated with Fix$^+$ (mean Fix$^+$ 125; OPS1475 RR 0.61, 95% CI [0.44, 0.85]; OPS1474 RR 0.47, 95% CI [0.33, 0.68]) (**Figure 3.2*C***). Plants inoculated with OPS1475 had a mean shoot dry biomass of 0.24 g, similar to those inoculated with Fix$^-$ (mean 0.27 g, ns difference of 0.03, 95% CI [-0.07, 0.13]), while plants inoculated with OPS1474 had an intermediate shoot dry biomass (mean 0.42 g), more per plant than Fix$^-$ (0.15 g more, 95% CI [0.04, 0.25]), and less per plant than Fix$^+$ (0.21 g less, 95% CI [0.10, 0.32]) (**Figure 3.2*D***).

As OPS1475 fixed no nitrogen, and OPS1474 fixed at an intermediate rate, this demonstrates that the *fixC*::ΩSp^r insertion in OPS1475 resulted in a non-fixing strain (Fix$^-$), whilst the ΩSp^r insertion between *nifA* and *fixX* in OPS1474 resulted in an intermediate-fixing strain (Fix^{int}).

As OPS1474 was shown to be Fix^{int}, we used the same plants to determine the nodule size of the Fix$^+$, Fix^{int}, and Fix$^-$ strains. The ten largest nodules per plant were measured to ensure that all selected nodules were at a similar developmental stage. Nodule size data was combined with that of one additional experiment conducted by Annet Westhoek with two or three replicates per strain. Nodule size was analysed in a linear mixed-effects model, with strain as a fixed effect, and experiment as a random effect.

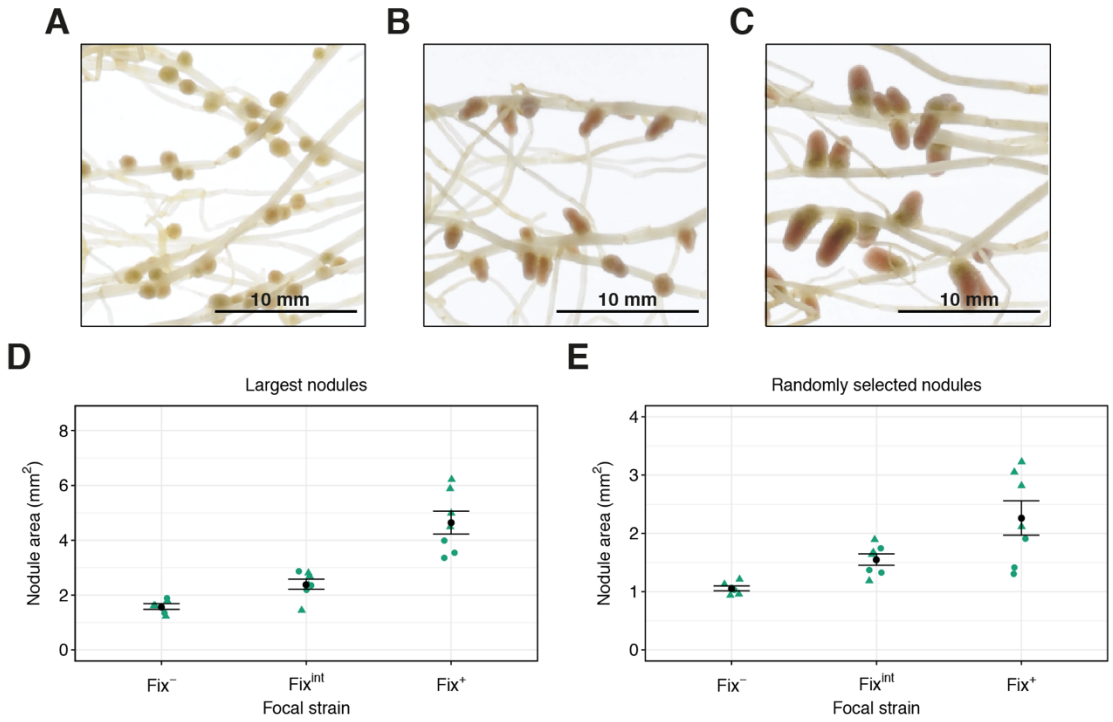


Figure 3.3 – Nodule sizes in single inoculations. (A) Fix$^-$ nodules were pale and small. (B) Fix^{int} nodules were pink and of intermediate size. (C) Fix$^+$ nodules were dark pink and large. (A-C) Scale bars indicate 10 mm, images shown are representative of six to seven biological replicates. Fix^{int} nodules were larger than Fix$^-$ and smaller than Fix$^+$ when the (D) largest and (E) randomly selected nodules were measured (note the scale difference). (D, E) Points represent an average of ten nodules per independent replicate plant. Circles and triangles indicate data from independent experiments. Black points indicate the overall mean with one standard error. Plants were grown for 27-28 days, n = 6-7.

In single inoculations, nodule size was dependent on the identity of the strain within ($F_{2,16}$ = 35.439, $p < 0.001$). Fix^{int} nodules (mean 2.37 mm^2) were larger than Fix$^-$ nodules (mean 1.52 mm^2, a difference of 0.85 mm^2, 95% CI [0.07, 1.58]), and smaller than Fix$^+$ nodules (mean 4.62 mm^2, a difference of 2.25 mm^2, 95% CI [1.53, 2.97]) (**Figure 3.3*D***). When ten randomly selected nodules were measured, similar results were seen. Nodule size was dependent on the strain within ($F_{2,16}$ = 13.985, $p < 0.001$). Fix^{int} nodules (mean 1.52 mm^2) were larger than Fix$^-$ nodules (mean 0.99 mm^2, a difference of 0.53 mm^2, 95% CI [0.06, 0.99]), and smaller than Fix$^+$ nodules (mean 2.23 mm^2, a difference of 0.71 mm^2, 95% CI [0.27, 1.16]) (**Figure 3.3*E***). Fix^{int} nodules were pink (**Figure 3.3*B***), darker than those of Fix$^-$ (**Figure 3.3*A***) and paler than those of Fix$^+$ (**Figure 3.3*C***). This demonstrates that in

single inoculations, Fix^{int} nodules have an intermediate size and colour phenotype, in addition to their intermediate nitrogen fixation rates.

3.4.2.2 Stability of the ΩSp^r insertion

It is possible that the intermediate nitrogen fixation and nodule size phenotype of Fix^{int} is due to the loss of the ΩSp^r cassette in a portion of the cells, allowing for undisrupted expression of genes in the operon. To determine the stability of the ΩSp^r cassette *in planta*, we assessed the ability of Fix^{int} (OPS1474) colonies from nodule extracts to grow on selective media containing spectinomycin.

One pea seed was potted per 500 mL pot containing medium vermiculite and inoculated with ~1 x 10^8 cells of OPS1474. Four replicate plants and one water control per time point (14, 21 and 28 dpi) were grown in a fully randomised design. At harvest, nodule extracts were plated, then 100 colonies per plant were patch-plated onto non-selective media (TY agar with streptomycin and nystatin), and selective media (TY agar with streptomycin, spectinomycin and nystatin), and colony growth observed after three days.

Harvest point (dpi)	Average patched colony growth on non-selective media	Average patched colony growth on selective media
14	100.0 %	99.5 %
21	100.0 %	99.5 %
28	100.0 %	100.0 %

Table 3.2 – Average growth of 100 patched colonies per plant on non-selective media (TY + St + Nys) and selective media (TY + St + Sp + Nys). n = 4 plants per time point.

For plants harvested at 14 and 21 dpi, almost all (99.5%) patched colonies were able to grow on selective media, and at 28 dpi all colonies were able to grow on selective media

(**Table 3.2**), which indicates that the colonies were still resistant to spectinomycin. This demonstrates that the ΩSp^r cassette is stable in OPS1474 *in planta* over time.

3.4.3 Assessment of conditional sanctioning by nodule size

3.4.3.1 Nodule size in double inoculations

To assess whether plants sanction Fix^{int} when it is not the best available strain, we measured the size of nodules when Fix^{int} was competing against Fix$^+$, itself, or Fix$^-$.

Plants were co-inoculated with a 1:1 ratio of unmarked Fix^{int} and *gusA*-marked Fix$^+$ (Rlv3841 *gusA*), itself, or *gusA*-marked Fix$^-$ (OPS0365). *gusA*-marked Fix$^+$ and Fix$^-$ were used so that, once plants were harvested, GusA staining could be used to visually distinguish between the different strains[135]. One pre-germinated pea seed was potted per 500 mL pot containing a 1:1 mixture of silver sand and fine vermiculite and inoculated with a 1:1 mixture of strains totalling 1 x 10^4 cells. Eight replicate plants and four water controls were grown for 28 days in a fully randomised design. At harvest, cells were sequentially stained for GusA and CelB[135]. The ten largest nodules per strain per plant were measured to ensure that all selected nodules were at a similar developmental stage. Nodule size data were combined with that of two additional experiments conducted by Annet Westhoek, each with five replicates per strain. Nodule size was analysed as a function of strain in a linear mixed-effects model with the identity of the competing strain (Fix$^+$, Fix^{int}, and Fix$^-$) as a fixed effect, and experiment as a random effect. The size of Fix^{int} nodules when competing against Fix$^+$ was compared with the size of Fix$^-$ nodules using a two-sample t-test. The proportion of nodules formed by Fix^{int} was analysed as a function of the identity of the competing strain (Fix$^+$, Fix^{int}, and Fix$^-$), with a generalised linear model using a quasibinomial error distribution.

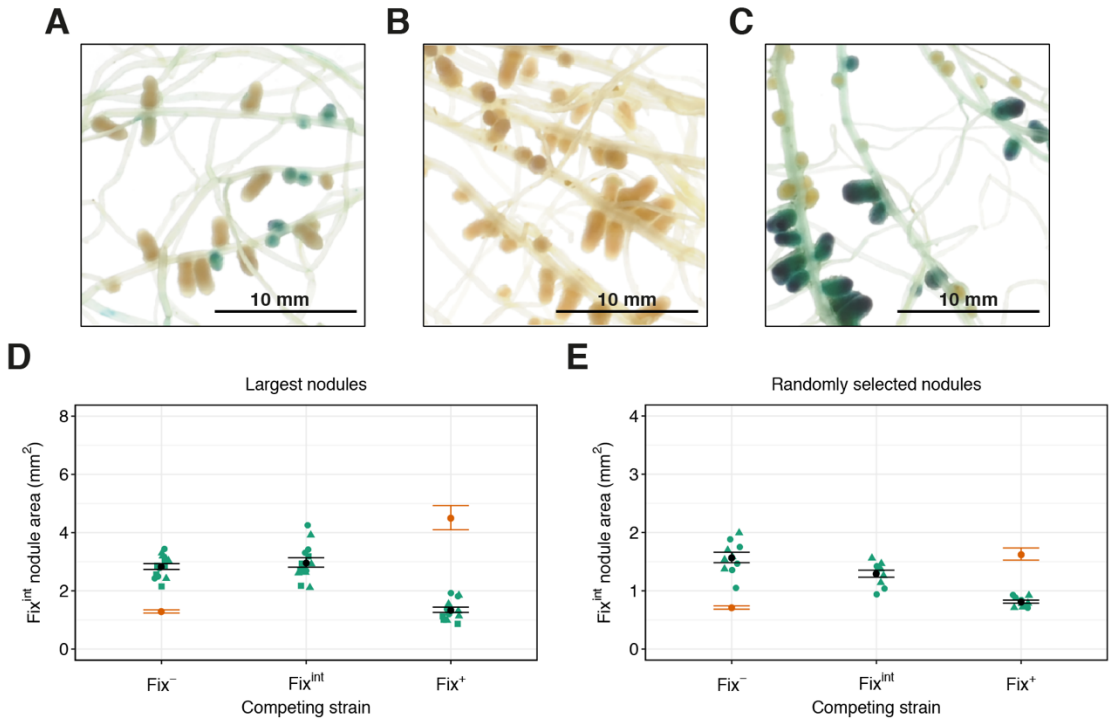


Figure 3.4 – Nodule sizes in double inoculations. (A) Fix^{int} nodules (unmarked) were large when competing against Fix^{-} (*celB*-marked, blue). (B) Fix^{int} nodules (unmarked) were large when competing against itself. (C) Fix^{int} nodules (unmarked) were small when competing against Fix^{+} (*celB*-marked, blue). (A-C) Scale bars indicate 10 mm, and images shown are representative of ten biological replicates. Fix^{int} nodules were smaller when competing against Fix^{+}, compared to against itself or Fix^{-} (D) when the largest nodules were measured and (E) when randomly selected nodules were measured (note the scale difference). Points represent an average of ten nodules per strain per independent replicate plant. Circles, triangles, and squares indicate data from independent experiments. Black points indicate the overall mean with one standard error. Orange points indicate overall mean with one standard error for the competing strain on the same plants. Plants were grown for 28 days. (D, E) n = 10.

In double inoculations, the size of Fix^{int} nodules was highly dependent on the identity of the competing strain ($F_{2,36} = 60.532$, $p < 0.001$). Fix^{int} nodules were significantly smaller when competing against Fix^{+} (mean 1.36 mm^2), compared to against itself (mean 2.99 mm^2, a difference of 1.63 mm^2, 95% CI [1.31, 1.95]) or Fix^{-} (mean 2.85 mm^2, a difference of 1.49 mm^2, 95% CI [1.17, 1.81]). When Fix^{int} competed against Fix^{+}, Fix^{int} nodules were as small as Fix^{-} nodules ($t_{26} = 0.54658$, $p = 0.589$). (**Figure 3.4*D***). When ten randomly selected nodules were measured, similar results were seen. Again, the size of Fix^{int} nodules was highly dependent on the identity of the competing strain ($F_{2,27} = 36.047$, $p < 0.001$). Fix^{int} nodules were significantly smaller when competing against Fix^{+} (mean

0.81 mm^2), compared to against itself (mean 1.29 mm^2, a difference of 0.48 mm^2, 95% CI [0.31, 0.65]) or Fix^- (mean 1.57 mm^2, a difference of 0.76 mm^2, 95% CI [0.58, 0.93]) (**Figure 3.4*E***). This demonstrates conditional sanctioning, as Fix^{int} nodules are large only when it is the best available strain on the plant.

Fix^{int} typically formed around half of the total nodules on the plant independent of the identity of the competing strain ($F_{1,26} = 0.1325$, $p = 0.7188$; against Fix^- mean = 44.86%, 95% CI [40.77, 49.03]; against Fix^+ mean = 48.80%, 95% CI [42.41, 55.24]), indicating that it was equally effective at forming nodules.

3.4.3.2 Nodule size in triple inoculations

As measurements of nodule size supported the hypothesis of conditional sanctioning in double inoculations, we assessed whether resources are still only allocated to the best available strain when all three strains were present on the same plant.

Plants were inoculated with a 1:1:1 triple inoculation of *gusA*-marked Fix^+ (Rlv3841 *gusA*), Fix^{int}, and *celB*-marked Fix^- (OPS0366). Fix^+ and Fix^- were *gusA*- or *celB*-marked so that, after harvesting, GusA and CelB sequential staining could be used to visually distinguish between the different strains[135]. One pre-germinated pea seed was potted per 500 mL pot containing a 1:1 mixture of silver sand and fine vermiculite and inoculated with a 1:1:1 mixture of strains totalling 1 x 10^2 cells per pot. Eight replicate plants and four water controls were grown for 27 days in a fully randomised design. At harvest, cells were sequentially stained for GusA and CelB[135]. The ten largest nodules per strain per plant were measured to ensure that all selected nodules were at a similar developmental stage. Nodule sizes were analysed using a linear mixed-effects model, with the identity

of the competing strain (Fix$^+$, Fix^{int}, and Fix$^-$) as a fixed effect, including plant as a random effect as the nodule sizes of all three strains on each plant were measured.

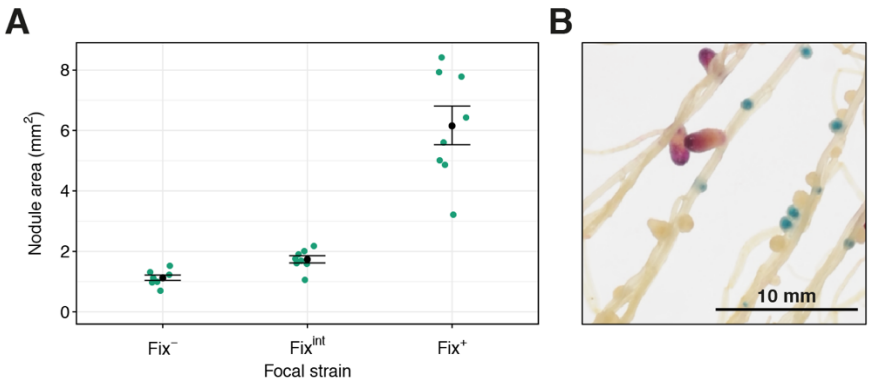


Figure 3.5 – Nodule sizes in triple inoculations. (A) Fix^{int} nodules were as small as Fix$^-$ nodules, and smaller than Fix$^+$ nodules. Points represent an average of ten nodules per strain per independent replicate plant. Black points indicate the overall mean with one standard error. Plants were grown for 27-28 days, n = 8. (B) Fix$^+$ nodules (*gusA*-marked, magenta) are large, while Fix^{int} nodules (unmarked) were as small as Fix$^-$ nodules (*celB*-marked, blue). Scale bar indicates 10 mm. Image shown is representative of eight biological replicates.

In triple inoculations, nodule size was dependent on the identity of the strain within ($F_{2,14}$ = 63.332, $p < 0.001$). Fix^{int} nodules (mean 1.74 mm^2) were similar in size to Fix$^-$ nodules (mean 1.13 mm^2, ns difference of 0.61 mm^2, 95% CI [-0.34, 1.56]), but significantly smaller than Fix$^+$ nodules (mean 6.17 mm^2, difference of 4.43 mm^2, 95% CI [3.48, 5.38]) **(Figure 3.5)**. This further demonstrates conditional sanctioning, as only the best available strain, Fix$^+$, had large nodules.

3.4.4 Generation of fluorescently marked strains for competition assays

3.4.4.1 Marking strains with GFP and mCherry

As we had found support for conditional sanctioning of the Fix^{int} strain using measurements of nodule size, we wanted to further investigate this process. To enable a further range of experiments, we marked strains with GFP and mCherry fluorescent

proteins, so that nodules containing different strains could be distinguished without the damaging process of GusA and CelB sequential staining.

The fluorescent proteins sfGFP[180] and mCherry3[181] were added to Fix^{int} and Fix^{-} by mini-Tn*7* insertion into the chromosome[149,151]. The GFP and mCherry donor plasmids (pOPS1526 and pOPS1531) were conjugated into Fix^{int} (OPS1474) and Fix^{-} (RU3940) using pTNS3[149] and selected for on TY agar with streptomycin and gentamycin. Fix^{+} (Rlv3841) was already marked with GFP (OPS1339) and mCherry (OPS1341) in this way[108].

Four individual colonies per conjugation were checked by colony PCR. Two colonies of each recipient strain and each donor plasmid were used as negative controls. Primers oxp2327 and oxp3060 were used to amplify a ~250 bp region from the right Tn*7* end into the chromosome, and primers oxp2328 and oxp3061 were used to amplify a ~500 bp region from the left Tn*7* end into the chromosome. All tested colonies showed the expected band size for the left flank, and all but one colony for Fix^{-} and GFP showed the expected band size for the right flank. A correct colony for each strain was stocked with Fix^{int} GFP as OPS2268, Fix^{int} mCherry as OPS2269, Fix^{-} GFP as OPS2270, and Fix^{-} mCherry as OPS2271.

3.4.4.2 In planta testing of GFP- and mCherry-marked strains

We tested the GFP- and mCherry-marked strains *in planta* to determine whether they could nodulate plants and be used to identify the strain within a nodule.

Plants were inoculated with single inoculations of unmarked Fix$^+$ (Rlv3841), Fix^{int} (OPS1474), or Fix$^-$ (RU3940); GFP-marked Fix$^+$ (OPS1339), Fix^{int} (OPS2268), or Fix$^-$ (OPS2270); or mCherry-marked Fix$^+$ (OPS1341), Fix^{int} (OPS2269), or Fix$^-$ (OPS2271). Two pea seeds were potted per 500 mL pot containing medium vermiculite and inoculated with ~1 x 10^8 cells per seed. After seven days plants were thinned to one per pot. In one experiment, three replicate plants for each Fix$^+$ strain and two water controls were grown for 28 days in a fully randomised design. In another experiment, two replicate plants for each Fix^{int} and Fix$^-$ strain, and two water controls were grown for 28 days in a fully randomised design.

Figure 3.6 shows fluorescence microscopy images of all nine strains and the water control *in planta*. The unmarked strains (Fix$^+$ Rlv3841, Fix^{int} OPS1474, and Fix$^-$ RU3940) had nodules which did not show either GFP or mCherry fluorescence. The GFP-marked strains (Fix$^+$ OPS1339, Fix^{int} OPS2268, and Fix$^-$ OPS2270) had nodules which looked similar to those of their parent strain and showed GFP fluorescence whilst not showing mCherry fluorescence. The mCherry-marked strains (Fix$^+$ OPS1341, Fix^{int} OPS2269, and Fix$^-$ OPS2271) also had nodules which looked similar to those of their parent strain and showed mCherry fluorescence whilst not showing GFP fluorescence. No nodules were observed on water controls. This demonstrates that the fluorescence emitted by the GFP- and mCherry-marked strains can be detected in nodules, and that the markers can be used to distinguish between strains when labelled with either GFP or mCherry.

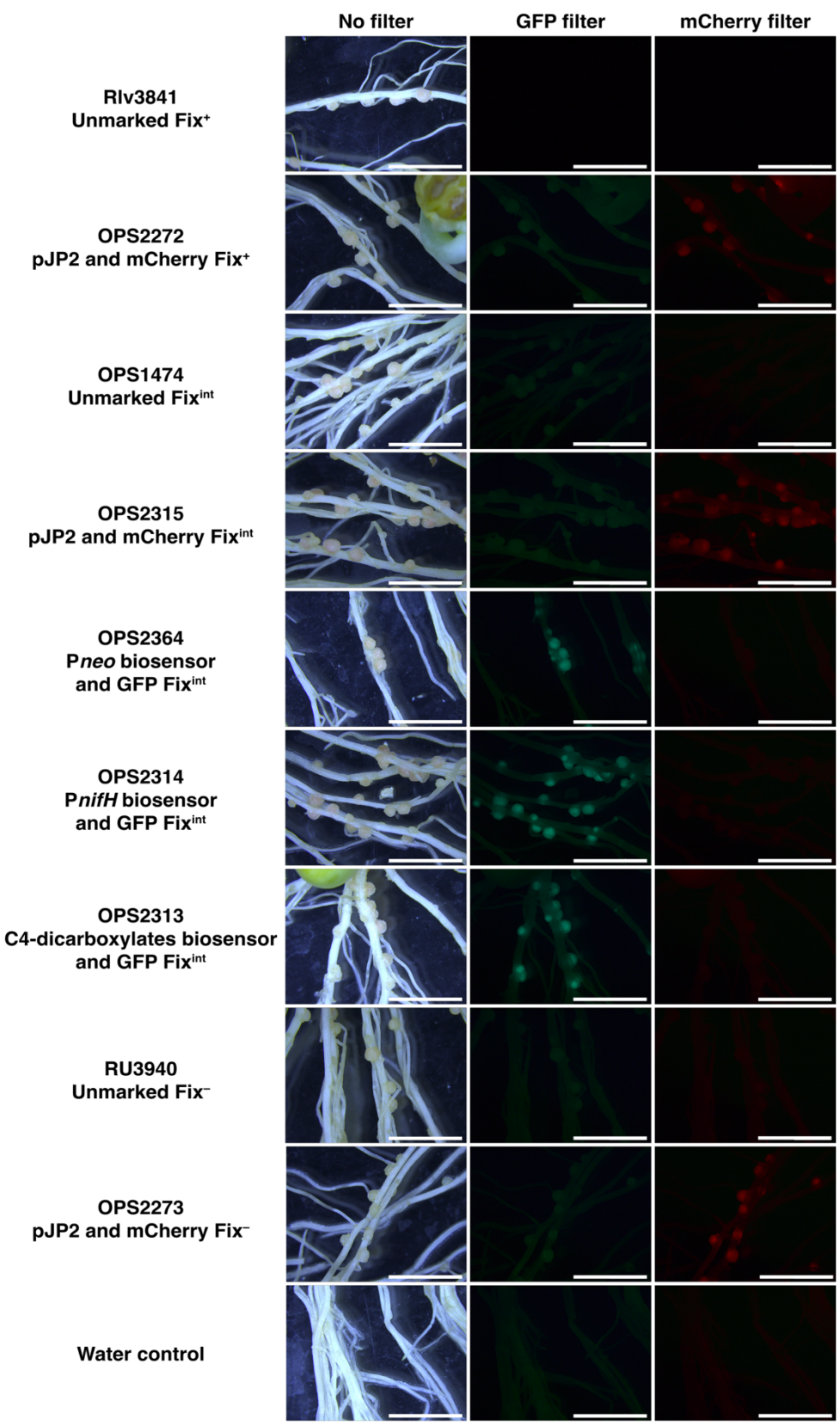


Figure 3.6 – Nodules formed by unmarked, GFP-marked, or mCherry-marked Fix^{+}, Fix^{int}, and Fix^{-} strains. Images are bright-field (left), or fluorescence with a GFP filter (centre) or mCherry filter (right). Scale bar indicates 10 mm. Plants were harvested at 28 dpi. Images shown are representative of three biological replicates (Fix^{+} strains) or two biological replicates (all other strains).

3.4.4.3 Growth rate of GFP- and mCherry-marked strains

As the GFP- and mCherry-marked strains were to be used in competition assays, we ensured that the markers would not affect the results of competition assays by determining that the markers did not impact the growth of strains. This was achieved by measuring the generation times of all the marked strains, and their unmarked parental strains, via growth curves. One replicate of each strain was measured in three independent experimental repeats. Generation times were analysed as a function of strain using a linear model, excluding water controls as these showed no growth.

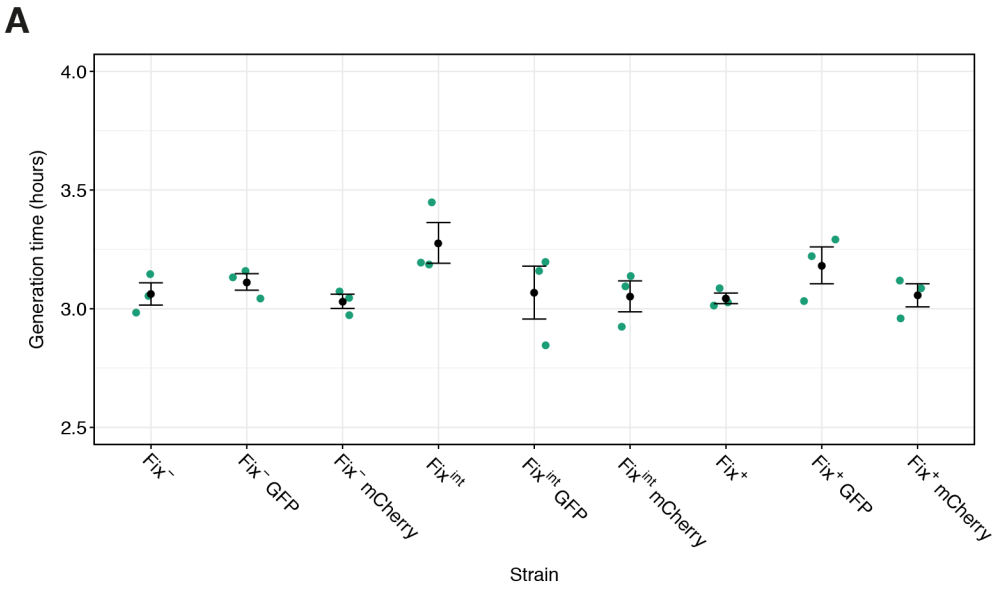


Figure 3.7 – The effect of fluorescent marker gene on generation time. (A) Generation time did not vary significantly among unmarked, GFP-marked, or mCherry-marked Fix^{+}, Fix^{int}, or Fix^{-}. Points represent independent replicates. Black points indicate the overall mean with one standard error, n = 3.

Growth curves showed a mean generation time of 3.10 hours, which did not vary significantly among strains ($F_{8,18}$ = 1.6004, p = 0.1936, **Figure 3.7**). This demonstrates that the GFP and mCherry fluorescent markers did not have a deleterious effect on the growth of any of the strains under the *in vitro* conditions tested.

3.4.4.4 Nitrogen fixation of GFP- and mCherry-marked strains

As the GFP and mCherry fluorescent markers did not impact the growth rate of strains *in vitro*, we then compared the effects of the markers on the nitrogen fixation rates of the strains. It was important to determine that the markers did not have a differential effect on the fixation rates of the strains, as any differences seen in competition assays should be due to differences in the parental strain (Fix^+, Fix^{int} or Fix^-), not the marker used.

Plants were inoculated with single inoculations of GFP-marked Fix^+ (OPS1339), Fix^{int} (OPS2268), or Fix^- (OPS2270); or mCherry-marked Fix^+ (OPS1341), Fix^{int} (OPS2269), or Fix^- (OPS2271). One pea seed was potted per 500 mL pot containing medium vermiculite and inoculated with ~1 x 10^8 cells per pot. In two independent experiments, four replicate plants per treatment and four water controls were grown for 28 days in a fully randomised design. Acetylene reduction rates were measured, the number of nodules counted, and water controls excluded as these formed no nodules. Acetylene reduction rates were analysed as a function of parent strain (Fix^+, Fix^{int} and Fix^-) and marker (GFP and mCherry) using a linear model.

The GFP and mCherry markers did not affect the nitrogen fixation rate of strains (non-significant effect of marker, $F_{1,44} = 0.421$, $p = 0.520$), so this term was removed from the model. The Fix^{int} strains fixed 0.011 μmol ethylene $nodule^{-1}$ $hour^{-1}$ less (95% CI [0.006, 0.015]) than Fix^+, a 39.04% reduction, and 0.017 μmol ethylene $nodule^{-1}$ $hour^{-1}$ more (95% CI [0.012, 0.021]) than Fix^- (**Figure 3.8*A***). Plants inoculated with the GFP-marked strain were similar in colour to those inoculated with the mCherry-marked strain for each strain pair of Fix^-, Fix^{int}, and Fix^+ (**Figure 3.8*B***). This demonstrates that the GFP and

mCherry fluorescent markers did not differentially affect the nitrogen fixation rates of any of the strains, and that even when marked, Fix^{int} fixes at an intermediate rate.

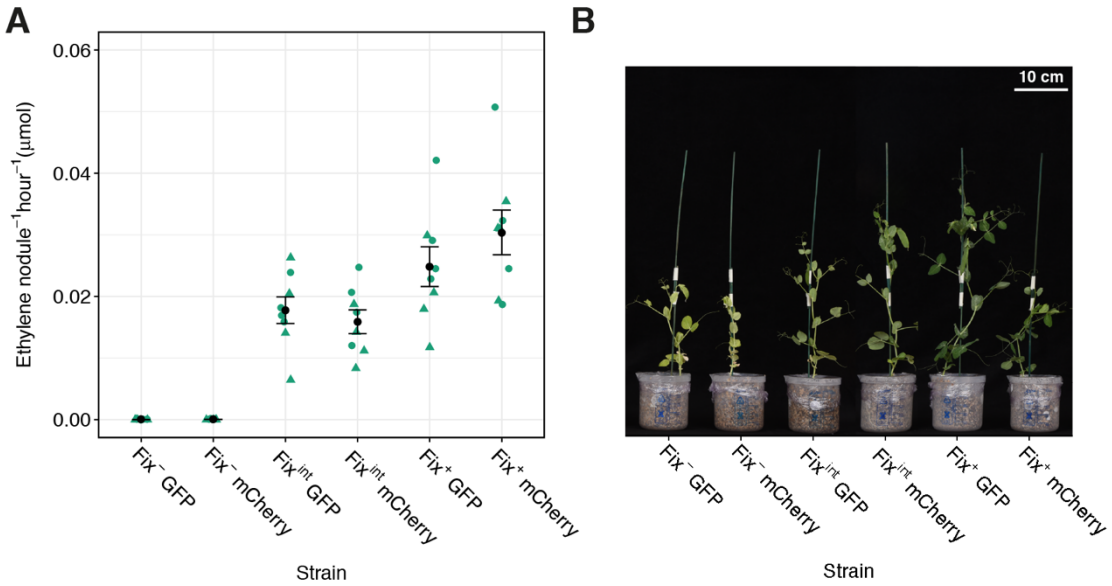


Figure 3.8 – The effect of fluorescent marker gene on nitrogen fixation rates and plant appearance. (A) The GFP and mCherry markers did not affect the nitrogen fixation rates of strains. Points represent an average of three technical replicates per independent replicate plant. Circles and triangles indicate data from independent experiments. Black points indicate the overall mean with one standard error. (B) Plants inoculated with the GFP-marked strain were similar in colour to those inoculated with the mCherry-marked strain for each strain pair of Fix$^-$, Fix^{int}, and Fix$^+$. (A, B) Plants were grown for 28 days, n = 8.

3.4.4.5 Nodulation and fitness of GFP- and mCherry-marked strains

As the GFP and mCherry fluorescent markers did not impact the growth rate *in vitro* or nitrogen fixation rates of strains, we then compared their ability to nodulate the host plant and their fitness when in competition. The ability of a strain to form nodules indicates both its *in vivo* growth rate, and its ability to nodulate. It was important to determine that the markers did not impact these characteristics, to ensure any differences seen in competition assays are due to differences in the parental strain (Fix$^+$, Fix^{int} or Fix$^-$), not the marker used.

Plants were co-inoculated with a 1:1 ratio of GFP-marked Fix$^+$ (OPS1339) and mCherry-marked Fix$^+$ (OPS1341). One pre-germinated pea seed was potted per 500 mL pot containing a 1:1 mixture of silver sand and fine vermiculite and inoculated with a 1:1 mixture of strains totalling 1 x 10^4 cells per pot. In two independent experiments, five replicate plants per treatment and one water control were grown for 28 days in a fully randomised design. At harvest, ten nodules per strain were selected for rhizobia extraction, serial dilutions and drop plate counts. The proportion of nodules formed by the GFP-marked strain was analysed with a generalised linear model using a quasibinomial error distribution. The number of CFUs per nodule was analysed in a linear model as a function of marker (GFP or mCherry). We attempted to include a random effect of plant to account for the fact that nodules for each marker were sampled from the same plant, but the model would not converge, so this term was excluded.

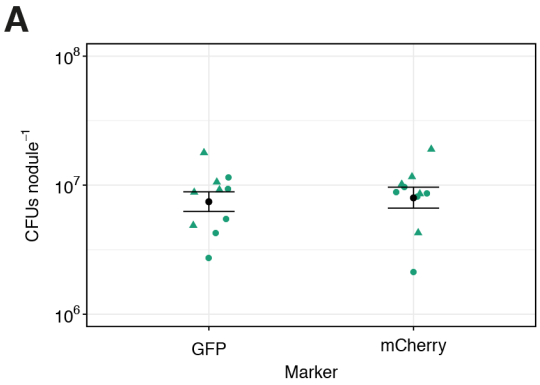


Figure 3.9 – The effect of fluorescent marker gene on CFUs per nodule. (A) The number of CFUs per nodule did not vary between the GFP- and mCherry-marked Fix$^+$ strains. Points represent an average of five technical replicates per strain per independent replicate plant, with each technical replicate consisting of ten combined nodules. Circles and triangles indicate data from independent experiments. Black points indicate the overall mean with one standard error. Plants were grown for 28 days, n = 10.

In double inoculations, the number of CFUs per nodule did not vary between the GFP- and mCherry-marked strains ($F_{1,18}$ = 0.105, p = 0.750, **Figure 3.9*A***). Additionally, the GFP-marked strains occupied a mean of 54.11% of nodules (95% CI [49.98, 58.18]). This

demonstrates that the GFP and mCherry fluorescent markers did not have different effects on the nodulation ability or fitness of strains.

3.4.5 Generation of *lux*-marked strains for competition assays

3.4.5.1 Marking strains with pJP2 plasmids

As measurements of nodule size supported the hypothesis of conditional sanctioning, we assessed whether this size difference could be explained by changes in the supply of carbon to the strain. To measure this, luminescent biosensor plasmids were added to Fix^{int} strains, which luminesce in response to the expression of nitrogen fixation and carbon transport genes. These biosensors contain *luxCDABE* genes under the control of a specific promoter, so that *luxCDABE* is co-expressed with other genes at key stages of nodule development and light is emitted. The P*neo* biosensor luminesces constitutively, as an indicator of background transcription activity, and the P*nifH* biosensor luminesces when nitrogenase genes are expressed during nodule development[143]. The sucrose and C4-dicarboxylates biosensors indicate carbon supply to the strain, as they luminesce when the sucrose transporter and the *dctA* transporter (which transports C4-dicarboylates) are transcribed[143].

The plasmid pJP2, the parental plasmid from which the biosensor plasmids are derived, was added to the competitor strains (Fix^{+}, Fix^{int} and Fix^{-}) to control for the potential cost to the strain of carrying a plasmid. To distinguish between strains, the biosensor-marked Fix^{int} strains were also marked with GFP, and the pJP2-marked competitor strains with mCherry.

To make the mCherry and pJP2 double-marked strains, mCherry3[181] (pOPS1531) was added to pJP2-marked Fix$^+$ (RU1416) and Fix$^-$ (LMB640) by mini-Tn*7* insertion into the chromosome[149,151]; and pJP2 was added to mCherry-marked Fix^{int} (OPS2269) by triparental mating. To make the GFP and biosensor double-marked Fix^{int} strains, sfGFP[180] (pOPS1526) was added to Fix^{int} marked with the sucrose biosensor (OPS2137) or the C4-dicarboxylates biosensor (OPS2136) by mini-Tn*7* insertion; and the P*neo* (pIJ11282) or P*nifH* (pLMB640) biosensors were added to GFP-marked Fix^{int} (OPS2268) by triparental mating. Conjugations were selected for on TY agar with the appropriate antibiotics.

Four individual colonies per conjugation were checked by colony PCR. Two colonies of each recipient strain and each donor plasmid were used as negative controls. For mini-Tn*7* insertions, the insertion was confirmed using primers oxp2327 and oxp3060 to amplify a ~250 bp region from the right Tn*7* end into the chromosome, and primers oxp2328 and oxp3061 to amplify a ~500 bp region from the left Tn*7* end into the chromosome. For triparental mating, presence of the plasmid was confirmed using primers oxp0345 and pIJ11268rv to amplify a 399 bp region in pJP2; or primers pIJ11268fw and pIJ11268rv to amplify a 1,584 bp region in pLMB640 or a 1,011 bp region in pIJ11282.

One correct colony for each strain was stocked with mCherry and pJP2 double-marked Fix$^+$, Fix^{int}, and Fix$^-$ as OPS2272, OPS2315, and OPS2273 respectively; and GFP and biosensor double-marked Fix^{int} as OPS2364 (P*neo*), OPS2314 (P*nifH*), OPS2278 (sucrose), and OPS2313 (C4-dicarboxylates).

3.4.5.2 In planta testing of pJP2-marked strains

We then tested pJP2-marked strains *in planta* to determine whether they could nodulate plants and be used to distinguish between nodules containing different strains. Additionally, the luminescence of nodules was measured to test if the luminescence of strains containing the *lux* cassette could be observed.

Plants were inoculated with single inoculations of unmarked Fix^+ (Rlv3841), Fix^{int} (OPS1474), or Fix^- (RU3940); mCherry and pJP2 double-marked Fix^+ (OPS2272), Fix^{int} (OPS2315), or Fix^- (OPS2273); or GFP and biosensor double-marked Fix^{int} (P*neo* OPS2364, P*nifH* OPS2314, and C4-dicarboxylates OPS2313). Two pea seeds were potted per 500 mL pot containing medium vermiculite and inoculated with ~1 x 10^8 cells per seed. Two replicate plants for each strain and two water controls were grown for 14 days in a fully randomised design. After seven days plants were thinned to one per pot.

Figure 3.10 shows fluorescence microscopy images of all nine strains and the water control *in planta*. The unmarked strains (Fix^+ Rlv3841, Fix^{int} OPS1474, and Fix^- RU3940) had nodules which did not show either GFP or mCherry fluorescence. Nodules of the mCherry and pJP2 double-marked strains (Fix^+ OPS2272, Fix^{int} OPS2315, and Fix^- OPS2273); looked similar to those of their parent strain and showed mCherry fluorescence whilst not showing GFP fluorescence. Nodules of the GFP and biosensor double-marked Fix^{int} strains (P*neo* OPS2364, P*nifH* OPS2314, and C4-dicarboxylates OPS2313) also looked similar to those of their parent strain and showed GFP fluorescence whilst not showing mCherry fluorescence. No nodules were observed on water controls.

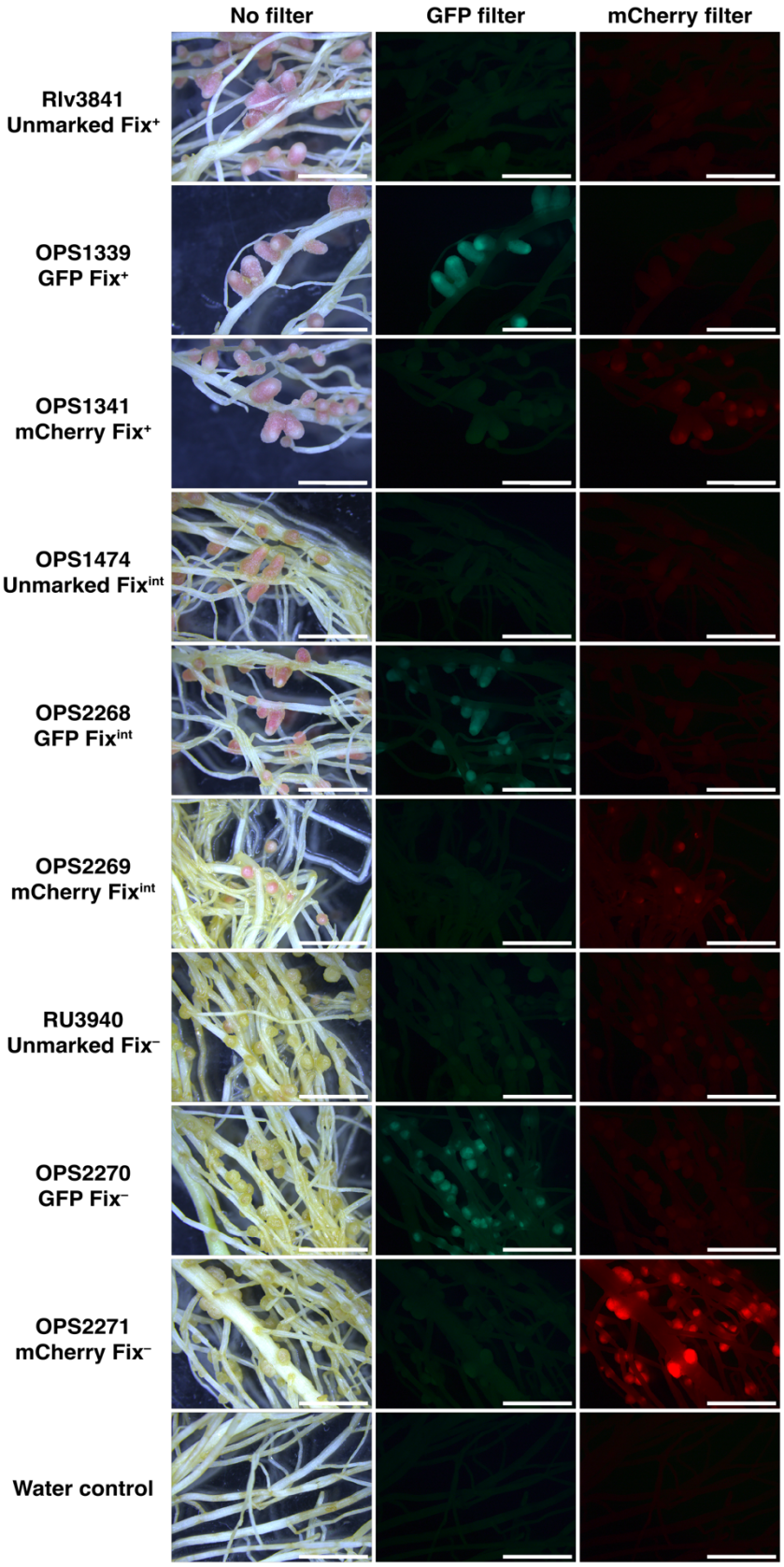


Figure 3.10 – Nodules formed by unmarked, GFP and biosensor double-marked Fix^{int}, or mCherry and pJP2 double-marked Fix^{+}, Fix^{int} and Fix^{-} strains. Images are bright-field (left), or fluorescence with a GFP filter (centre) or mCherry filter (right). Scale bar indicates 10 mm. Plants were harvested at 14 dpi. Images shown are representative of two biological replicates.

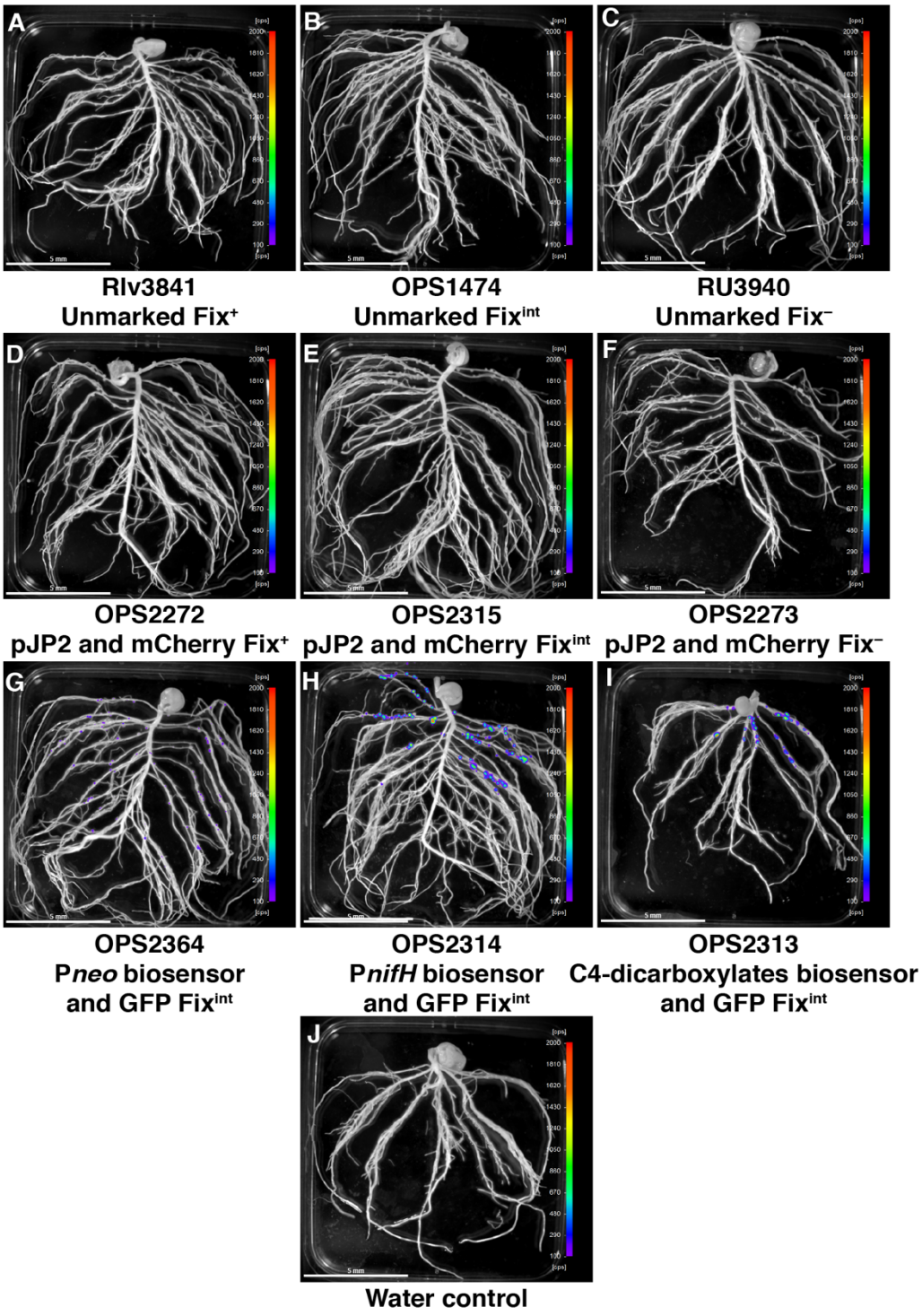


Figure 3.11 – Luminescence from nodules formed by unmarked, GFP and biosensor double-marked Fix^{int}, or mCherry and pJP2 double-marked Fix^{+}, Fix^{int} and Fix$^{–}$ strains. Plants were harvested at 14 dpi and imaged with a NightOWL CCD camera. Root systems are shown in greyscale, with luminescence from 100 – 2000 cps in colour as indicated by the heat map legend. Scale bar indicates 5 cm. Images shown are representative of two biological replicates.

NightOWL images showing luminescence of all nine strains and the water control are shown in **Figure 3.11**. Luminescence over 100 cps was detected in nodules of the double-marked GFP and biosensor Fix^{int} strains (P*neo* biosensor OPS2364 **Figure 3.11*G***, P*nifH* biosensor OPS2314 **Figure 3.11*H***, and C4-dicarboxylates biosensor OPS2313 **Figure 3.11*I***). No luminescence was detected in the water control (**Figure 3.11*J***), or in the nodules of the unmarked strains (**Figure 3.11*A-C***), or the mCherry and pJP2 double-marked strains (**Figure 3.11*D-F***).

This demonstrates that the fluorescence emitted by the double-marked strains can be detected in nodules, and that the strains can be distinguished when labelled with either GFP or mCherry. Additionally, luminescence of the biosensors can be detected, so these double-marked strains can be used to demonstrate gene expression or carbon supply to the strain.

The Fix^{int} strain, double-marked with GFP and *luxCDABE* under the promotion of the sucrose transporter promoter (OPS2278), was not included in this test as the conjugation was not completed in time. However, this strain was included in further experiments under the assumption that its fluorescence and luminescence would be detected in a similar manner to the other strains.

3.4.5.3 Growth rate of pJP2-marked strains

As the pJP2-marked strains were to be used in competition assays, we ensured that the markers would not affect the results of competition assays by determining that the markers did not impact the growth of strains. This was achieved by measuring the generation times of all the marked strains, and their unmarked parent strains, via growth

curves. One replicate of each strain was measured in three independent experimental repeats. Generation times were analysed as a function of strain using a linear model, excluding water controls as these showed no growth.

Growth curves showed a mean generation time of 3.06 hours, which did not vary significantly among strains ($F_{9,20}$ = 0.1306, p = 0.9982, **Figure 3.12**). This demonstrates that the pJP2 markers did not have a deleterious effect on the growth of any of the strains under the *in vitro* conditions tested.

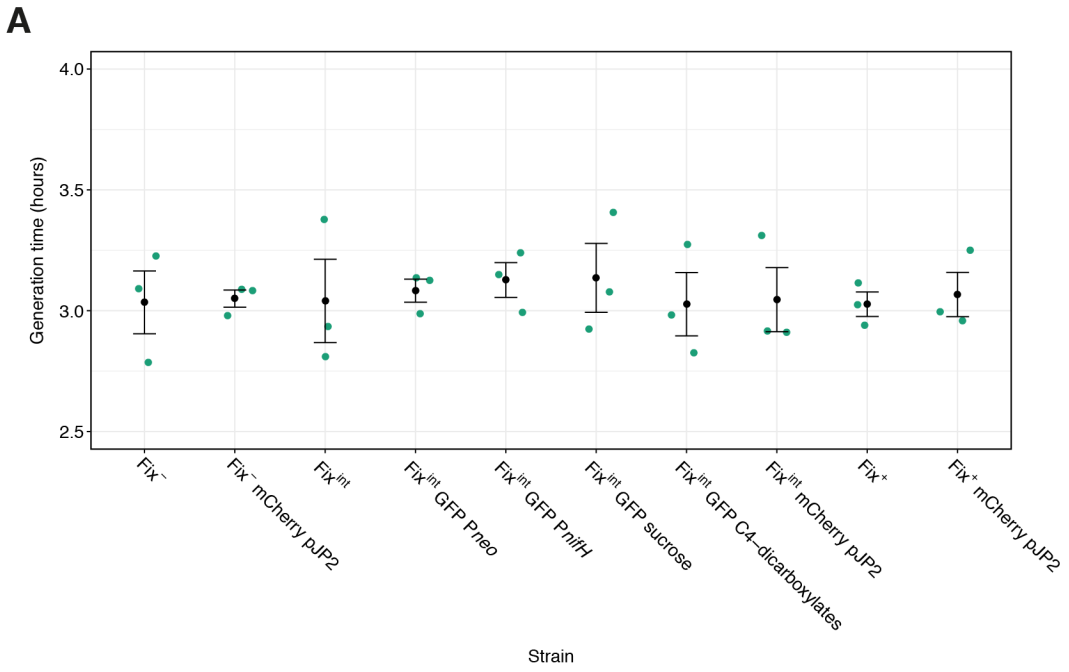


Figure 3.12 – **The effect of fluorescent marker genes and biosensor plasmids on generation time.** (A) Generation time did not vary significantly among unmarked, unmarked Fix$^+$, Fix^{int}, or Fix$^-$; mCherry and pJP2 double-marked Fix$^+$, Fix^{int}, or Fix$^-$; or GFP and biosensor double-marked Fix^{int}. Points represent independent replicates. Black points indicate the overall mean with one standard error, n = 3.

3.4.6 Assessment of conditional sanctioning by carbon supply

As measurements of nodule size supported the hypothesis of conditional sanctioning, we tested whether this size difference could be a result of changes in carbon supply to the

nodule. Luminescent biosensors were used to visualise the expression of genes involved in nitrogen fixation and carbon transport in Fix^{int} when competing against Fix^{+} or Fix^{-}.

Plants were co-inoculated with a 1:1 ratio of a GFP and luminescent biosensor double-marked Fix^{int} (P*neo* OPS2364, P*nifH* OPS2314, sucrose OPS2278, or C4-dicarboxylates OPS2313) and mCherry and pJP2 double-marked Fix^{+} (OPS2272) or Fix^{-} (OPS2273). One pre-germinated pea seed was potted per 500 mL pot containing a 1:1 mixture of silver sand and fine vermiculite and inoculated with a 1:1 mixture of strains totalling 1 x 10^4 cells per pot. In two independent experiments, three replicate plants per treatment per time point and four water controls per time point were grown for 12 or 20 days in a fully randomised design. The percentage of intermediate-fixing nodules emitting light (thus expressing *luxCDABE*) was calculated and analysed in separate generalised linear models for each biosensor (P*neo*, P*nifH*, C4-dicarboxylates or sucrose), with a quasibinomial error distribution where the fixed effects are the identity of the competing strain (Fix^{+} or Fix^{-}), time point (12 or 20 dpi), and their interaction.

In the early stages of nodule development (12 dpi), when only a few nodules had started to express nitrogenase genes (14.6%, 95% CI [5.9, 31.9] **Figure 3.13*A***), carbon was supplied to almost all Fix^{int} nodules (**Figure 3.13*C, D***). This did not depend on the identity of the competing strain (ns difference with odds ratio [OR] 2.27 for C4-dicarboxylates, 95% CI [0.08, 56.77] **Figure 3.13*C***; OR 0.45 for sucrose, 95% CI [0.03, 7.57] **Figure 3.13*D***). The percentage of luminescent Fix^{int} nodules for the constitutive P*neo* biosensor also did not depend on the identity of the competing strain (OR 1.03, 95% CI [0.31, 3.37] **Figure 3.13*A***), indicating similar transcription activity for both strains.

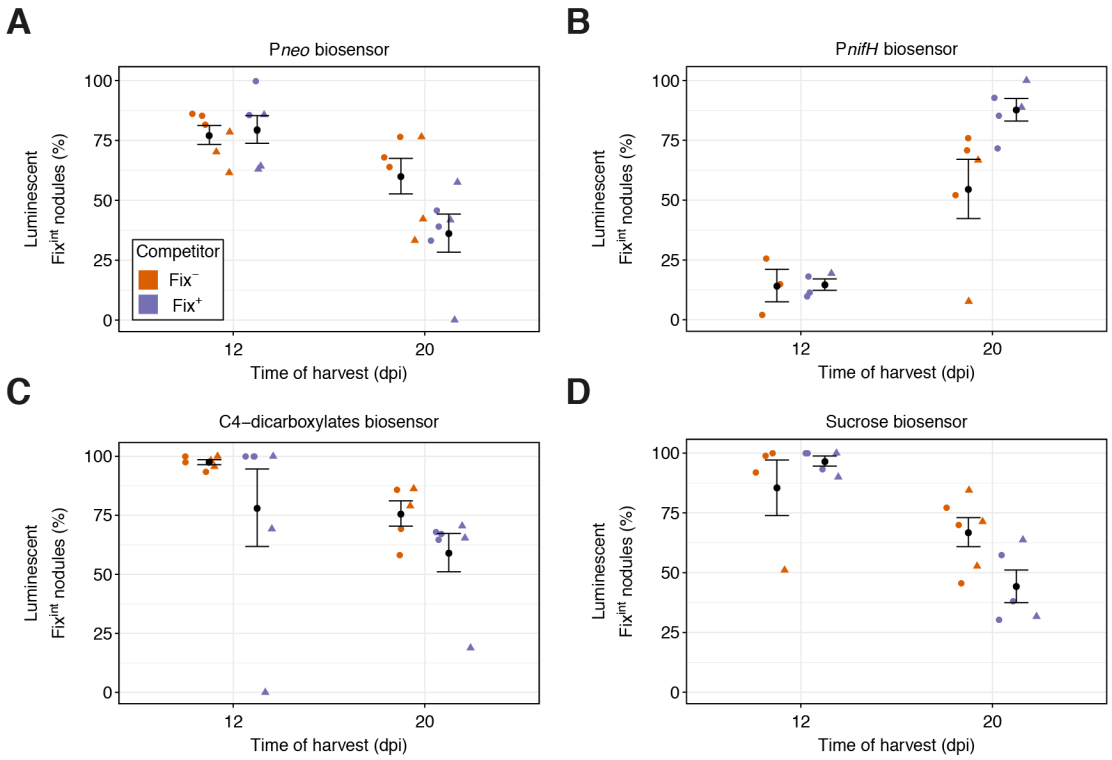


Figure 3.13 – The effect of the competing strain and harvest point on Fix^{int} biosensor luminescence. At 12 dpi, the percentage of luminescent Fix^{int} nodules did not depend on the co-inoculated strain for any of the luminescent biosensors: P*neo* (A) P*nifH* (B), C4-dicarboxylates (C), and sucrose (D). At 20 dpi, when most nodules were fixing nitrogen (B), Fix^{int} marked with the sucrose biosensor had a significantly higher percentage of luminescent nodules when competing against Fix^{-}, than against Fix^{+} (D). (A-D) Points represent independent replicate plants. Circles and triangles indicate data from independent experiments. Black points represent the overall mean with one standard error. (A) n = 6, (B) n = 3-5, (C) n = 5-6, (D) n = 4-6.

At a later developmental stage (20 dpi), most nodules were expressing nitrogenase genes (71.8%, 95% CI [59.3, 81.6] (Fig. 3*B*)), which would allow the plant to discriminate between strains based upon their fixation ability. Here, a smaller percentage of Fix^{int} nodules showed active carbon transport when competing against Fix^{+} compared to when competing against Fix^{-}. The difference was significant in the case of sucrose (OR 2.81, 95% CI [1.22, 6.43] **Figure 3.13*D***), but not quite significant in the case of C4-dicarboxylates (OR 1.99, 95% CI [0.81, 4.93] **Figure 3.13*C***). Additionally, at this time point a smaller percentage of Fix^{int} nodules showed luminescence for the P*neo* biosensor when competing against Fix^{+}, compared to those competing against Fix^{-} (OR 1.91, 95% CI [1.04, 3.50] **Figure 3.13*A***). In contrast, a larger percentage of Fix^{int} nodules showed

luminescence for the P*nifH* biosensor when competing against Fix^+, compared to those competing against Fix^- (OR 0.25, 95% CI [0.08, 0.76] (**Figure 3.13*B***).

Results at 20 dpi further demonstrate conditional sanctioning, as carbon transport to and transcription activity of Fix^{int} is highest when it is the best available strain on the plant.

3.4.7 Assessment of conditional sanctioning by acetylene reduction

The size of Fix^{int} nodules, and the carbon supply to these nodules, was conditional on the identity of the competing strain, supporting the hypothesis of conditional sanctioning. We then looked at how this conditional carbon supply might affect the nitrogen fixation rate of Fix^{int}. This was assessed by acetylene reductions of plants after removing all nodules containing competing strains, leaving only nodules containing the Fix^{int} strain.

Plants were co-inoculated with a 1:1 ratio of mCherry-marked Fix^{int} (OPS2269) and GFP-marked Fix^+ (OPS1339), Fix^{int} (OPS2268), or Fix^- (OPS2270). One pre-germinated pea seed was potted per 500 mL pot containing a 1:1 mixture of silver sand and fine vermiculite and inoculated with a 1:1 mixture of strains totalling 1 x 10^4 cells per pot. In two independent experiments, four replicate plants per treatment and four water controls were grown for 28 days in a fully randomised design. At harvest, all GFP-marked nodules containing the competitor strains were removed, leaving only the mCherry-marked Fix^{int} nodules for acetylene reduction. The number of nodules was also counted. Rates were analysed using a linear mixed-effects model, with the identity of the competing strain as a fixed effect and experiment as a random effect.

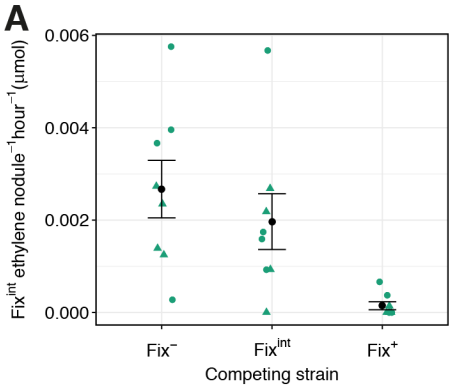


Figure 3.14 – The effect of the competing strain on Fix^{int} nitrogen fixation rates. (A) Fix^{int} fixed more nitrogen per nodule when competing against Fix^{-}, or itself, than against Fix^{+}. Points represent an average of three technical replicates per independent replicate plant. Circles and triangles indicate data from independent experiments. Black points indicate the overall mean with one standard error. Plants were grown for 28 days, n = 8.

The per-nodule nitrogen fixation rate of Fix^{int} was dependent on the identity on the competing strain ($F_{2,20}$ = 7.2161, p = 0.0044). Fix^{int} became effectively non-fixing when competing against Fix^{+} (0.146 x 10^{-3} µmol ethylene nodule^{-1} hour^{-1}, 95% CI [0.000 x 10^{-3}, 1.874 x 10^{-3}]) fixing less nitrogen than when competing against a differently-marked version of itself (1.820 x 10^{-3} µmol ethylene nodule^{-1} hour^{-1} less, 95% CI [0.480 x 10^{-3}, 3.159 x 10^{-3}]) or Fix^{-} (2.524 x 10^{-3} µmol ethylene nodule^{-1} hour^{-1} less, 95% CI [1.185 x 10^{-3}, 3.864 x 10^{-3}] **Figure 3.14*A***). This further demonstrates conditional sanctioning, as the nitrogen fixation rate of Fix^{int} nodules was highest when it was the best available strain on the plant.

3.4.8 Assessment of conditional sanctioning by rhizobial fitness

Finally, as the size, carbon supply to, and nitrogen fixation rate of the Fix^{int} nodules were conditional on the identity of the competing strain, supporting the hypothesis of conditional sanctioning, we looked at whether this difference in resource allocation would be reflected in differences in rhizobial fitness within the nodule. The number of

reproductive rhizobia in Fix^{int} nodules was determined when competing against Fix^{+}, Fix^{-}, or a differently-marked version of itself.

Plants were co-inoculated with a 1:1 ratio of GFP-marked Fix^{int} (OPS2268) and mCherry-marked Fix^{+} (OPS1341), Fix^{int} (OPS2269), or Fix^{-} (OPS2271), or mCherry-marked Fix^{int} (OPS2269) and GFP-marked Fix^{+} (OPS1339), Fix^{int} (OPS2268), or Fix^{-} (OPS2270). One pre-germinated pea seed was potted per 1 L pot containing a 1:1 mixture of silver sand and fine vermiculite and inoculated with a 1:1 mixture of strains totalling 1 x 10^4 cells per pot. In three independent experiments, one replicate plant per treatment per time point and two water controls per timepoint were grown for 28 or 56 days in a fully randomised design. At harvest, ten nodules per plant were selected for rhizobia extraction, serial dilutions and drop plate counts. The number of CFUs per nodule was analysed in a linear model as a function of the identity of the competing strain, time point (28 and 56 dpi), and their interaction.

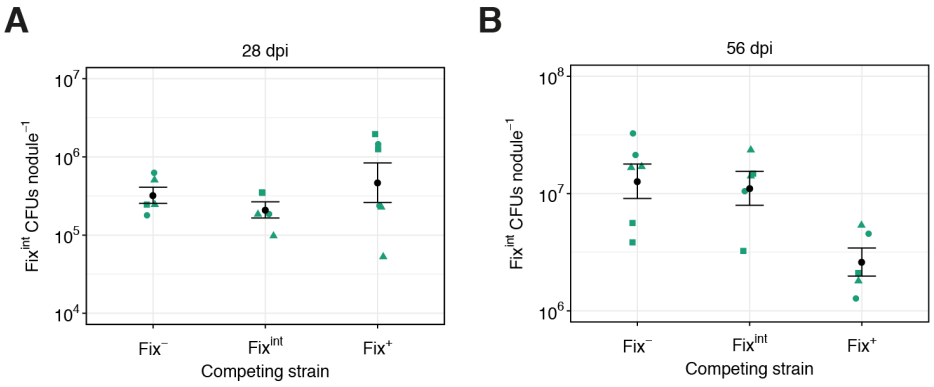


Figure 3.15 – The effect of the competing strain on CFUs per Fix^{int} nodule. (A) At 28 dpi Fix^{int} had similar numbers of CFUs per nodule when competing against Fix^{+}, compared to against itself, or Fix^{-}. (B) At 56 dpi Fix^{int} had significantly fewer CFUs per nodule when competing against Fix^{+}, compared to against itself, or Fix^{-}. (A-B) Points represent an average of five technical replicates per independent replicate plant, with each technical replicate consisting of ten combined nodules. Circles, triangles, and squares indicate data from independent experiments. Black points indicate the overall mean with one standard error. Plants were grown for 28 or 56 days, n = 5-6.

The number of CFUs per Fix^{int} nodule was dependent on the identity of the competing strain and the age of the plant at harvest (interaction $F_{2,26} = 4.612$, $p = 0.019$). At 28 dpi Fix^{int} had similar numbers of CFUs per nodule when competing against Fix^{+}, compared to against itself (ns difference of 6.357×10^5, 95% CI [-6.259×10^6, 7.530×10^6]) or against Fix^{-} (ns difference of 5.071×10^5, 95% CI [-6.387×10^6, 7.401×10^6] **Figure 3.15*A***). However, at 56 dpi when competing against Fix^{+}, Fix^{int} had 0.303×10^7 CFUs per nodule, significantly fewer than when competing against itself (mean 1.324×10^7, 1.021×10^7 fewer, 95% CI [0.301×10^7, 1.741×10^7]) or Fix^{-} (mean 1.622×10^7, 1.318×10^7 fewer, 95% CI [0.629×10^7, 2.008×10^7] **Figure 3.15*B***). This further demonstrates conditional sanctioning, as the fitness of Fix^{int} rhizobia at a later developmental stage is highest when it is the best available strain on the plant.

3.5 Discussion

In this chapter we demonstrated that host plants can conditionally sanction their rhizobial partners based on their nitrogen fixation ability. We tested this by inoculating pea plants with different combinations of otherwise isogenic fixing, intermediate-fixing and non-fixing strains. In order to discriminate between the strains *in planta*, we used strains with chromogenic markers. We also marked and tested strains with fluorescent protein markers to allow the discrimination of strains without the damaging process of staining. We showed that intermediate fixers were only tolerated when a better strain was not available. Initially, carbon transport into intermediate-fixing rhizobia was similar, regardless of the identity of the competing strain. But as nodules developed, carbon transport into nodules and the growth of nodules containing an intermediate-fixing strain was reduced, resulting in smaller nodules with reduced nitrogen fixation, but only when a more effective strain was available to the plant. At an even later developmental stage, this reduction in resource allocation resulted in lower rhizobial fitness.

First, we characterised a strain which had an intermediate rate of nitrogen fixation, formed nodules of intermediate size, and also had an intermediate benefit for the plant as demonstrated by the resultant shoot dry biomass. We showed that the ΩSp^r cassette insertion in this strain was stable *in planta,* meaning that the reduced fixation phenotype was not simply caused by the loss of this cassette in some individuals within a nodule. The comparison of a genetically altered intermediate fixer with otherwise isogenic fixing and non-fixing strains allowed us to directly compare the host response to the nitrogen fixation abilities of its rhizobial partners. This is an improvement on previous studies

where naturally-occurring strains of rhizobia have been used[77,88,95], as we can distinguish the host response to nitrogen fixation from the host response to other strain traits.

To assess conditional sanctioning in the first instance, we measured the size of the intermediate fixer's nodules when competing against other strains, as sanctioning has been observed to affect nodule size[88,90,93,105,106]. In double inoculations, when the intermediate fixer was competing against a fixer, and therefore was the worst option for the plant, the nodule size of the intermediate fixer was reduced and was similar to a non-fixing nodule. This was further corroborated by results from triple inoculations of all three strains: here, the fixer was the best option for the plant and had large nodules, while the intermediate fixer and non-fixer had small nodules of similar size. In double inoculations, the intermediate fixer occupied around half of the nodules, showing that it can nodulate the host at the same frequencies as its competitors despite its reduced fixation ability.

We then demonstrated that carbon transport to the intermediate fixer was downregulated when a better strain was available. At an early developmental stage carbon transport was independent of the identity of the competing strain. This is likely because plants cannot discriminate amongst strains at this time point, as even nodules containing a superior strain provide little or no nitrogen at the start of nodule development, as indicated by the small percentage of nodules expressing P*nifH*. But as nodules developed, fewer intermediate-fixing nodules were receiving carbon, and in particular sucrose, when a better strain was available to the host. We would not expect to see no nodules receiving carbon even at this late stage of development, as pea plants nodulate continuously and therefore some nodules will always be at an earlier developmental stage. The increased energetic demand from maintaining a plasmid may affect the nitrogen-fixing ability of the strain, but to reduce the experimental impact of this the competing strains were

marked with empty plasmids. The intermediate fixer may also face an increased energetic demand from luminescing[182,183] which may affect its ability to fix nitrogen. However, the strain would still fix at an intermediate level when compared to the fixing and non-fixing strain, even if the absolute level differed.

Interestingly, the carbon transport experiments also showed that the number of nodules expressing P*nifH* was higher at a later time point when Fix^{int} was competing against a better strain. This suggests that in response to a low carbon supply, Fix^{int} nodules increase their expression of nitrogenase genes, possibly in an attempt to increase their nitrogen-fixing capacity in order to receive carbon in return. It is not clear if this increased fixation capacity correlates with an actual increase in nitrogen fixation at this time point, as this may be carbon-supply dependent. This could be further investigated by monitoring gene expression, carbon transport, and nitrogen fixation over a range of time points.

The reduction of carbon supply to the intermediate fixer also resulted in a reduced rate of nitrogen fixation, so that it effectively became non-fixing, demonstrating that conditional sanctioning can alter the nitrogen fixation rates of the least effective strains. These results were obtained by removing the nodules of the competing strains, which may have stressed the host plant and affected the results. However, the plants were kept moist, nodules removed swiftly within a few hours, and the order of plants to have their nodules removed was randomised. Therefore, even if plant stress affected nitrogen fixation rates in this experiment, we would not expect this to vary between treatment groups. An alternative to nodule picking is split-root systems. Here, the root system would be split into two pots with different inocula. This system has been used previously to study this symbiosis[94,119,126,184,185], and allows separate acetylene reductions of each half-root.

However, trial experiments with this system were not successful. The pea root systems rarely developed in both halves; those which did develop often only formed nodules on one root-half; and the overall nitrogen fixation rate per nodule was much lower than expected, indicating that at least in pea hosts, results from split-root experiments might not be reliable.

Conditional sanctioning also resulted in reduced rhizobial fitness at a later developmental stage, which may help prevent the spread of ineffective strains through the population[74,77,90]. Despite observed differences in carbon allocation by 20 dpi, and differences in nodule size and nitrogen fixation rate by 28 dpi, we did not observe a difference in the number of CFUs per nodule at 28 dpi, only at 56 dpi. The delayed response for fitness, as compared to nodule size and nitrogen fixation rate, may be explained as pea has indeterminate nodules, which contain two different forms of rhizobia. Nitrogen fixation is carried out by the larger bacteroids, which are formed by endoreduplication of the chromosome, and cannot reproduce outside of the nodule. In contrast the undifferentiated bacteria are smaller, do not fix nitrogen and can reproduce outside of the nodule[60]. In sanctioned nodules, the reduced nodule size at 28 dpi may be a result of sanctions restricting nodule development, with fewer rhizobia differentiating into the large non-reproductive bacteroids. However, for the undifferentiated bacteria, which can be cultured and counted, the impact of sanctioning and a reduced carbon supply seems not to affect their numbers at 28 dpi, but does by 56 dpi. The effects of sanctioning on non-culturable bacteroid numbers is further investigated in **Chapter 4**.

Our findings do not support the idea that strains are simply supplied with carbon in proportion to their fixation ability, as previously postulated[106]. Instead, the intermediate strain is only sanctioned when there is a better option available to the plant. This implies

that there must be a systemic mechanism to compare the effectiveness of different strains and adjust resource allocation accordingly. It cannot be determined from these results whether these comparisons are made at a per-nodule level, or within nodules. Within-nodule comparisons could allow hosts to discriminate amongst strains sharing a singular, mixed, nodule[96,107]. However, the effect of sanctioning and conditional sanctioning on strains within mixed nodules is further investigated in **Chapter 4**.

Overall, we have demonstrated that conditional sanctioning allows pea plants to discriminate between rhizobia that differ in their abilities to fix nitrogen, giving them remarkable control over their symbiotic partners. This could be an essential adaptive mechanism in systems where poor fixers are common. Furthermore, the effect of conditional sanctioning on rhizobial fitness may control the spread of ineffective strains through the population, helping to explain the stability of this symbiosis through evolutionary time.

Chapter 4 Quantifying sanctioning at a single-cell level using flow cytometry

4.1 Abstract

Plants host nitrogen-fixing rhizobia in specialised root nodules and provide the rhizobia with carbon in return for nitrogen. Plants can sanction nodules containing rhizobia which provide little or no nitrogen, resulting in smaller nodules containing fewer viable rhizobia, as determined by culturing nodule extracts. However, in indeterminate nodules, only undifferentiated rhizobia can reproduce. Rhizobia which terminally differentiate into bacteroids in order to fix nitrogen cannot reproduce, so cannot be counted by culturing nodule extracts. Limited histological evidence shows that sanctioned nodules have fewer or senesced bacteroids, but the effect of sanctioning on bacteroid numbers has yet to be quantified.

Here we inoculated pea (*Pisum sativum)*, which forms indeterminate nodules, with different combinations of fixing, intermediate-fixing, and non-fixing derivatives of the standard laboratory rhizobial strain *Rhizobium leguminosarum* bv. *viciae* 3841, which were otherwise isogenic. We used flow cytometry, a single-cell analysis technique, to test whether sanctioning affects the numbers of bacteroids per nodule, and whether sanctioning can act *within* a nodule.

Non-fixing nodules had fewer bacteroids, regardless of whether a better alternative strain was present. However, nodules containing the intermediate fixer had fewer bacteroids only when a better alternative was available for the host. This shows that the host plant can conditionally control the number of bacteroids, and therefore nodule development, depending on which strains are available. We also observed limited evidence for the host plant's ability to sanction *within* a mixed nodule containing more than one strain. Further study of mixed nodules could reveal whether sharing a nodule with a fixer would allow a non-fixer to avoid host sanctions.

4.2 Overview

The data that support the findings of this chapter are available in the Oxford University Research Archive (DOI: 10.5287/bodleian:zBpJPja9E).

4.3 Introduction

Legume plants can obtain nitrogen through a symbiosis with nitrogen-fixing bacteria, known as rhizobia. The plant hosts rhizobia in specialised root nodules and provides the rhizobia with carbon and other nutrients in return for nitrogen[14]. As discussed in **Chapter 3**, rhizobia could be selected to cheat by leaving the costly process of nitrogen fixation[82] to other strains, and instead invest in reproduction to gain higher fitness[83,84]. Plants can prevent wasting resources on poorly performing nodules by sanctioning such nodules[90,93,94,96,105–108,126]. However, precisely how sanctioning affects the different forms of rhizobia within a nodule, and whether sanctioning can be directed at only one strain when multiple strains share the same nodule, is less clear.

From an individual plant perspective, sanctioning prevents the host wasting resources on strains that provide little or no nitrogen[84,108]. The mechanism of sanctioning in legumes is unclear, but could be due to the plant withholding resources[108] or forcing the senescence of less effective strains[96]. Studies have shown that sanctioning can reduce the fitness of rhizobia, as fewer viable rhizobia are recovered from sanctioned nodules[90,93,94,105–108,126]. This has been shown both where the fixation ability of a strain has been genetically altered[105,107,108], and where nitrogen fixation has been reduced by the withholding of nitrogen gas[90,93,106]. From an evolutionary perspective, this sanction-induced reduction in rhizobial fitness may prevent the spread of ineffective strains through the population[74,77,90].

However, these studies of sanctioning have only quantified the rhizobia which can be cultured. This matters for hosts with indeterminate nodules, as not all rhizobia can be cultured[55,56,60–63]. In these nodules, rhizobia are dimorphic: they either remain small, do

not fix nitrogen, but retain the ability to reproduce, or they terminally differentiate into larger bacteroids in order to fix nitrogen[55,56,60–63], so losing their ability to reproduce. Therefore, while the culturing of nodule extracts and counting the resultant number of colony forming units (CFUs) has shown that sanctioning affects the number of reproductive bacteria recovered from indeterminate nodules[93,94,105,107,108,126], it does not reveal how the numbers of bacteroids are affected. In this work we use the term bacteria to refer to undifferentiated rhizobia in the nodule, bacteroids to refer to terminally differentiated rhizobia in the nodule, and rhizobia as a general term for all types of rhizobial cell.

Some evidence of the effect of sanctioning on bacteroids has been gathered from histological studies. Laguerre *et al.* (2012) co-inoculated two naturally-occurring strains of rhizobia and showed that when the host plant was nitrogen-limited, the more effective strain had more bacteroids per nodule when counted from histological sections[126]. However, as these strains were collected from the field, they may have differed in traits other than nitrogen fixation, such as nodulation competitiveness[100] and metabolic demands[63], so any observed host response could be due to a combination of these traits.

Daubech *et al*. (2017) co-inoculated a fixing strain and an otherwise-isogenic non-fixing strain, so avoiding the confounding factors that result from using natural strains. Here microscopy showed evidence for senescence of bacteroids in sanctioned nodules, although this study did not quantify the number of bacteroids per nodule[107]. Quantifying the number of bacteroids could aid our understanding of the timing of sanctioning and its effects on nodule development. Fewer bacteroids in sanctioned nodules could indicate

that these nodules have reduced development compared to their non-sanctioned counterparts.

Studying sanctioning at a single-nodule level could also reveal how the host plant responds to strains which differ in their nitrogen fixation ability when they reside in the same, mixed, nodule. Mixed nodules containing two[96,110–112] or more[113] strains form when multiple strains enter the initial root hair infection thread, which is the first stage in nodule formation[109]. If hosts are unable to discriminate between different strains within a mixed nodule and sanction accordingly, then mixed nodules could be a way for non-fixing strains to avoid sanctioning[97]. If a fixing strain provides enough nitrogen to avoid being sanctioned by the host plant, a non-fixing strain sharing the same nodule could gain higher fitness through investing in reproduction[83,84]. Daubech *et al*. (2017) provided quantitative evidence of sanctioning within a mixed indeterminate nodule. Here, culturing nodule extracts showed that a fixing strain had more CFUs per mixed nodule than an otherwise isogenic non-fixing strain[107]. Imaging further revealed that, within mixed nodules, the non-fixing strain had formed bacteroids, but these were senesced[107], in line with studies in determinate nodules[96]. Quantifying this phenomenon would give insights into the mechanism of sanctioning within mixed nodules, and how this compares to single-strain nodules.

Flow cytometry, a single-cell analysis technique, has the potential to shed light on the effects of sanctioning on both bacteria and bacteroids within nodules, as it would allow all cells within nodules to be quantified. In flow cytometry, cells flow past a light source, with scattered light giving information on cell size (forward scatter) and granularity (side scatter)[167]. As the bacteroids in indeterminate nodules are much larger than the bacteria[55],

the bacteroids have a larger forward scatter than bacteria[63], so flow cytometry can be used to separate[60,186] and quantify[63,187] the two populations within a nodule. Flow cytometry can also be used to distinguish cells that have different fluorescent markers[188–190], so if rhizobial strains carried different fluorescent proteins, the populations of bacteria and bacteroids for each strain could be quantified within mixed nodules.

Here, we tested the effects of sanctioning at a single-cell level using flow cytometry. We used pea (*Pisum sativum)* plants, inoculated with different combinations of otherwise isogenic fixing (Fix^+), intermediate-fixing (Fix^{int}), and non-fixing (Fix^-) derivatives of the standard laboratory rhizobial strain *Rhizobium leguminosarum* bv. *viciae* 3841 (Rlv3841), as in **Chapter 3**. The differing levels of nitrogen fixation were caused by the insertion of a Ω spectinomycin resistance (Sp^r) cassette, in the region between *fixX* and *nifA* for Fix^{int} [108], or in the *nifH* gene for Fix^-[105].

Strains were labelled with fluorescent proteins so that the occupant of each nodule could be identified, and so that strains could be distinguished from each other and from background signal in the flow cytometer. We tested several fluorescent proteins, deciding on green (superfolder green fluorescent protein, sfGFP[180]) and red (mCherry3[181]) as these could be clearly distinguished, both when visualising nodules under a microscope when trying to identify mixed nodules, and when in the flow cytometer. We also tested two promoters, and chose the strong constitutive consensus promoter J23104[191] as this expressed the fluorescent proteins in all rhizobial cells within the nodule.

We used these strains to: (1) quantify how sanctioning affects both bacteria and bacteroids at a single-cell level; and (2) test whether sanctioning can act within mixed nodules and

so prevent poor fixers from prospering by piggy-backing on fixing strains. First, we compared the numbers of bacteria and bacteroids in Fix^+ and Fix^- single-strain nodules, taken from plants which were singly-inoculated or co-inoculated with both strains. Second, we determined how sanctioning affects the number of bacteria and bacteroids in intermediate-fixing nodules (Fix^{int}) when co-inoculated with Fix^+, Fix^-, or a differently-marked version of itself. Finally, we tested whether sanctioning of Fix^- or Fix^{int} strains can be observed in mixed nodules, and how this affects both bacteria and bacteroids.

4.4 Results

4.4.1 Generation of fluorescently marked strains for competition assays

4.4.1.1 Generation of single-marked strains

To test how sanctioning affects the bacteria and bacteroid populations within nodules, we competed Fix^+, Fix^{int}, and Fix^- strains against each other and quantified the rhizobia within nodules using flow cytometry. We labelled strains with fluorescent proteins to distinguish between nodules containing different strains. Additionally, fluorescent proteins were used for flow cytometry to distinguish cells from background noise, and to distinguish between cells of each strain when mixed nodules were analysed.

Name	Abbreviation	Protein used	Strain number (Fix^+)
Blue fluorescent protein	BFP	mTagBFP[145]	OPS1344[189]
Cyan fluorescent protein	CFP	eCFP[192]	OPS1343[189]
Green fluorescent protein	GFP	sfGFP[180]	OPS1339[108]
mCherry (red fluorescent protein)	mCherry	mCherry3[181]	OPS1341[108]
Yellow fluorescent protein	YFP	eYFP[193]	OPS1340[189]

Table 4.1 – Fluorescent proteins used to mark strains.

We marked strains with a range of fluorescent proteins (**Table 4.1**) to test which would be the easiest to distinguish, both when selecting the nodules and in flow cytometry. Fix^+ (Rlv3841) was already labelled with these markers[108,189] by mini-Tn*7* insertion into the chromosome[149,151], as detailed in **Table 4.1.** These fluorescent proteins were under the promotion of J23104[191], a consensus strong constitutive promoter[194], which should be expressed in every rhizobial cell, including both bacteria and bacteroids.

4.4.1.2 Generation of double-marked strains

We were concerned that the J23104 constitutive promoter might not label all cells, particularly the bacteroids, as these have altered gene expression compared to bacteria[55], so we also tested a second promoter, Ps*nifH*[113]. This is a synthetic consensus version of the P*nifH* promoter, which drives the expression of the nitrogenase complex-encoding *nifHDK* genes in bacteroids[195], and therefore should label all bacteroid cells. To compare the performance of the two promotors, we double-marked rhizobia with both promoters, by generating a plasmid containing BFP controlled by Ps*nifH,* and adding this to the J23104 YFP strain (OPS1340).

The marker plasmid was assembled using Golden Gate cloning[130]. This involves using genetic modules, which, when digested by type IIs restriction enzymes, have sticky-ends, allowing the DNA fragments to assemble in the correct order within a vector[130,131]: the PU module (promoter with the ribosome binding site), SC module (ORF), and the T module (terminator). The PU module pOGG043 was used, containing Ps*nifH*[113]. The SC module pOGG129[146] contained mTagBFP[145]. The T module pOGG003 contained the pharmacia terminator[144]. The vector pOGG026 was used, which is broad host range and low copy number, and shown to be stable in rhizobia[144]. This vector contains Nm^r/Km^r in the vector backbone to select for the plasmid.

A Golden Gate reaction was carried out[131], the reaction mix transformed into Bioline α-Select Gold competent cells, and selected for on LB agar with kanamycin and X-gal. White colonies were selected, which indicates the displacement of *lacZα* in the cloning site of the vector, which would otherwise result in blue colonies[130].

Two individual colonies were checked by plasmid extraction and digestion with EcoRI. Both showed the expected band sizes of ~ 6 kb and ~ 1.1 kb. One plasmid was sent for sequencing with primers oxp0282 and oxp0283, and showed the correct sequence across the assembly junctions. The plasmid was stocked as pOPS1146 (**Figure 4.1**).

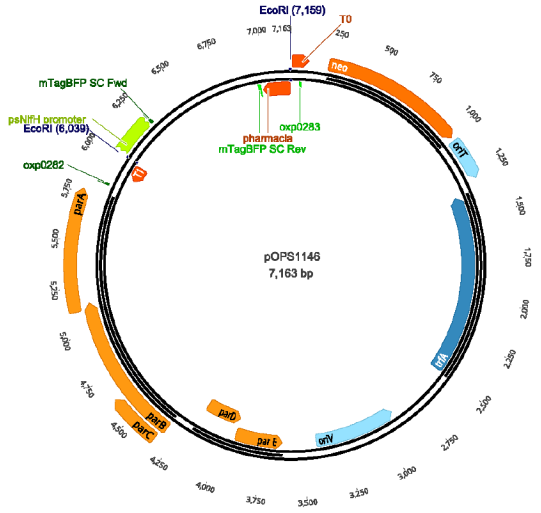


Figure 4.1 - Plasmid pOPS1146 containing BFP under Ps*nifH* promotion.

The BFP donor plasmid (pOPS1146) was then conjugated into the YFP-marked strain (OPS1340[189]) by triparental mating with the assistance of pRK2013[196]. Conjugations were selected for on TY agar with streptomycin and gentamycin, with added neomycin to select for the plasmid. Four individual colonies were checked by colony PCR. Two colonies of the recipient strain were used as a negative control, and two of the donor plasmid as a positive control. Primers oxp0282 and oxp0283 were used to amplify the assembly region in the donor plasmid (1388 bp). One correct colony was identified and stocked as OPS1906.

4.4.2 Comparing fluorescent proteins by flow cytometry

We then determined which fluorescent proteins would be best to use in competition assays, and which combinations of laser and detector would give maximum separation for each protein. We tested a range of fluorescent proteins with the J23104 promoter in the flow cytometer to determine which could be detected clearly, and which could be distinguished when in combination.

We grew cultures of unmarked Fix^+ (Rlv3841); and Fix^+ strains marked with GFP (OPS1339), YFP (OPS1340), mCherry (OPS1341), CFP (OPS1343), and BFP (OPS1344). Strains were measured in the flow cytometer, both individually and in all pairwise colour-combinations, excluding combinations with CFP, as it was suspected that this fluorescent protein would not be detected well by the flow cytometer. One biological replicate of each strain or combination of strains was sampled.

We demonstrated that the emission peaks of all tested fluorescent proteins could be detected using three laser and detector combinations. BFP and CFP could be detected using the 405 nm laser and the 456/51 nm detector (blue channel); YFP and GFP could be detected using the 488 nm laser and the 528/46 nm detector (green/yellow channel); and mCherry could be detected using the 532 nm laser and the 611/31 nm detector (red channel) (**Figure 4.2**).

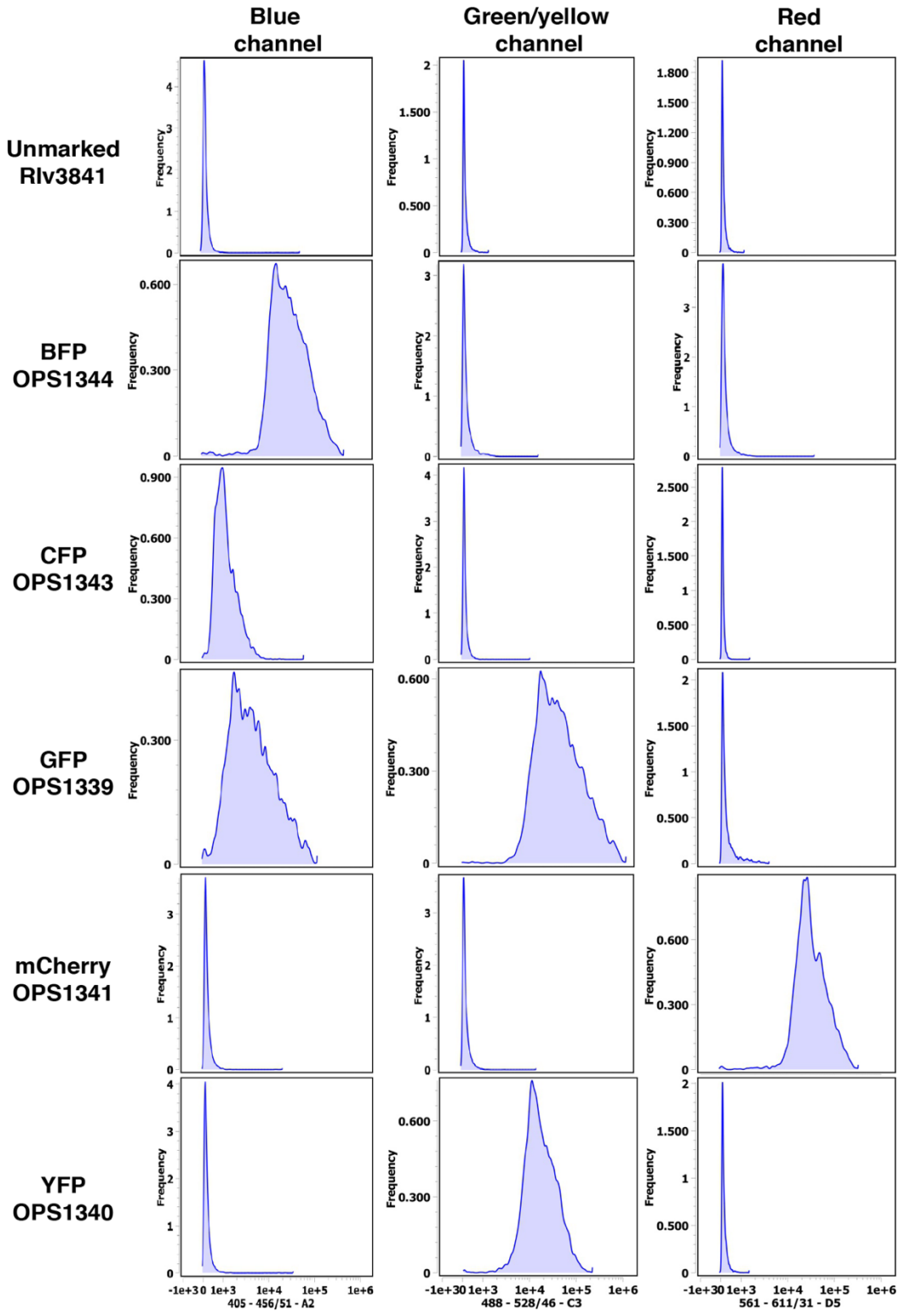


Figure 4.2 – Emission profiles of fluorescent proteins. Histograms show intensity of fluorescence in three detection channels: 405 nm laser and 456/51 nm detector (blue channel); 488 nm laser and 528/46 nm detector (green/yellow channel); and 532 nm laser and 611/31 nm detector (red channel). Spectra show histograms based on measurements of 5000 cells and are representative of three technical replicates per strain.

The expression profiles of each fluorescent protein are shown as histograms of the detected intensity in each of the three detection channels for individual cells (**Figure 4.2**). CFP was indeed poorly-detected, as its peak is not very different to that of the unmarked control, and we therefore decided not to use strains marked with CFP for competition assays. BFP, YFP and mCherry were expressed exclusively in their respective channels, meaning that all three could be used to label strains in competition. GFP had strong expression in the green/yellow channel and the blue channel, meaning GFP cannot be used in combination with YFP or BFP. However, GFP had no expression in the red channel, so can be used in combination with mCherry.

The expression profiles of fluorescent proteins in pairwise combinations are shown as heat maps of the detected intensity in two channels for individual cells (**Figure 4.3**). Here, unmarked cells are in the lower left quadrant, cells in the upper left quadrant were detected only in the channel listed on the y axis, cells in the lower right quadrant were detected only in the channel listed on the x axis, and cells in the upper right quadrant were detected in both listed channels. Negative values were caused by the process of compensation, where results are modified based on controls to reduce cross-channel detection[160] (as further explained in **Chapter 2**). For all tested combinations of strains, we see separate populations of cells in each channel, showing that all tested combinations of fluorescent proteins can be detected.

However, each sample has cells which are detected in both channels. This is likely due to two or more cells passing through the detector simultaneously, so that the flow cytometer cannot resolve them. This is likely to be a particular problem when trying to distinguish strains marked with different fluorescent proteins within the same sample, such as a

sample from a mixed nodule. However, this could potentially be accounted for in analyses by removing cells which appear to be marked by both fluorescent proteins.

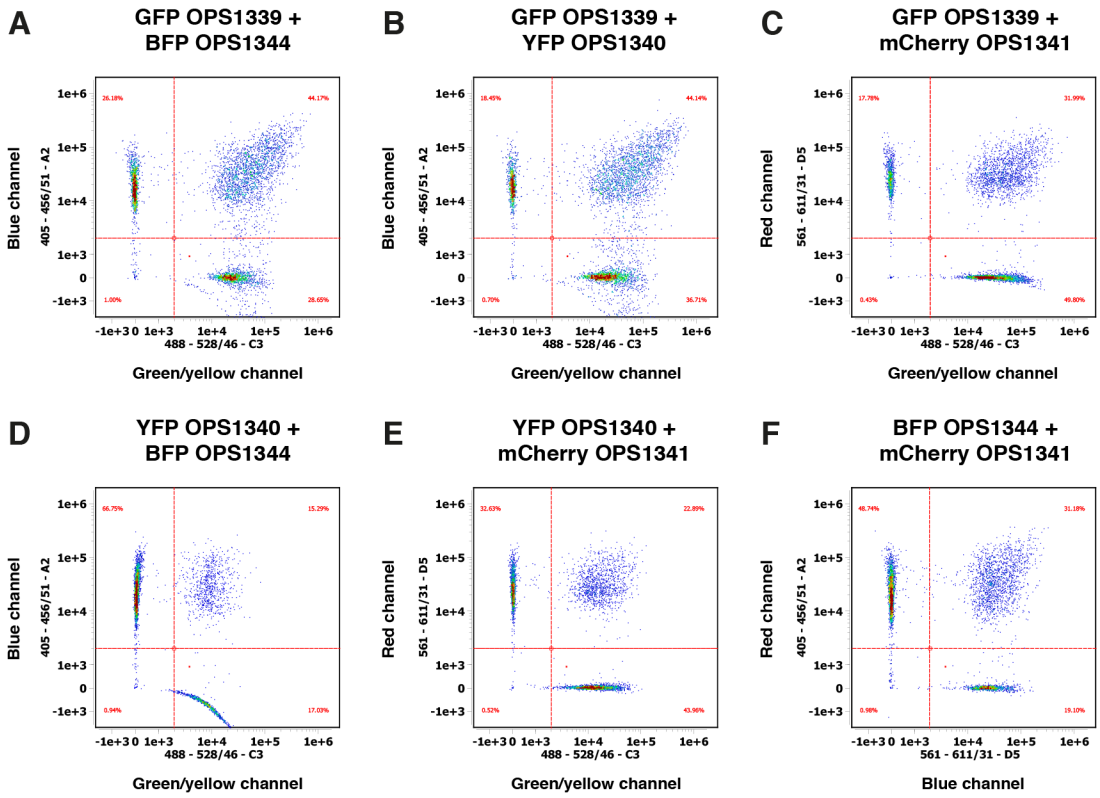


Figure 4.3 – Emission profiles of fluorescent proteins in combination. (A-F) Heat maps show the intensity of emissions detected in two channels for combinations of strains containing fluorescent proteins. 5000 cells were measured per sample, points represent individual cells, and graphs are representative of three technical replicates per strain.

Despite the flow cytometer being able to distinguish between many pairwise combinations of fluorescent proteins, we chose GFP and mCherry for competition assays. These are distinguishable using a fluorescence stereomicroscope, and when placed on a blue-light transilluminator and imaged with an orange filter, as in **Chapter 3**[108]. This would allow swift nodule selection and processing for flow cytometry experiments.

4.4.3 Comparing promoters for fluorescent proteins by flow cytometry

To test which promoter would best express fluorescent proteins for flow cytometry, we inoculated plants with strains which were double-marked with BFP under the promotion of Ps*nifH*, and YFP under the promotion of J23104. Nodules were analysed using a flow cytometer, to see which rhizobia cells were marked with which fluorescent protein.

Plants were inoculated with single inoculations of Fix$^+$ double-marked with Ps*nifH*-BFP and J23104-YFP (OPS1906). Two pre-germinated pea seeds were potted per 1 L pot containing medium vermiculite and inoculated with ~1 x 10^8 cells per seed. Four replicate plants and two water controls were grown for 28 days in a fully randomised design. After seven days plants were thinned to one per pot. At harvest, the ten largest nodules per strain per plant were selected for rhizobia extraction and flow cytometry, to ensure that all nodules were at a similar developmental stage.

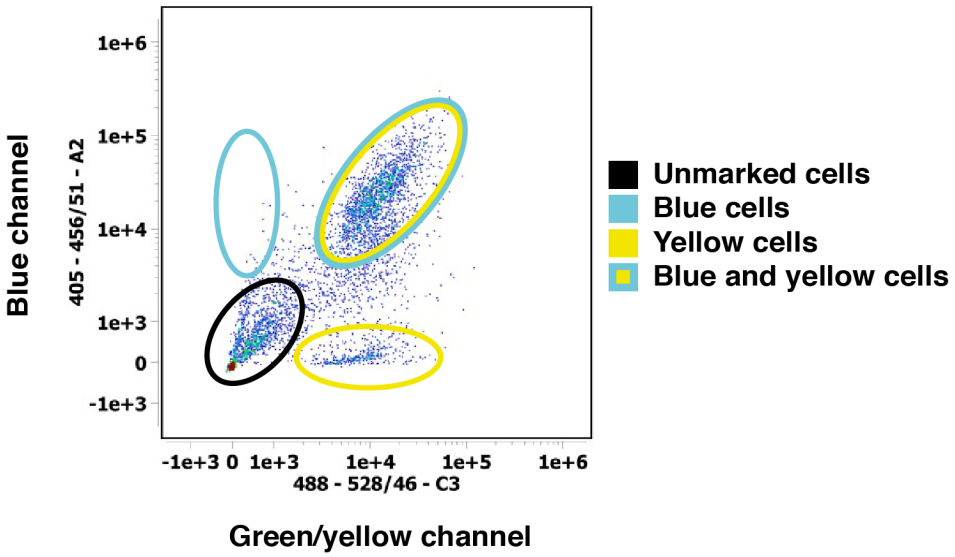


Figure 4.4 - Emission profile of a double-marked strain. Heat map showing the intensity of emissions detected in the blue channel and the green/yellow channel for a double-marked strain (OPS1906). 5000 cells were measured per sample, points represent individual cells, and the graph is representative of four biological replicates.

Figure 4.4 shows an example heat map when double-marked cells were measured using the blue and green/yellow channels. The figure shows dense patches of unmarked cells, yellow cells, and both blue and yellow cells; but few cells which are only blue. This shows that the J23104 promoter expressed YFP in all cells, but the Ps*nifH* promoter did not express BFP in all cells, resulting in cells which were marked with YFP but not BFP. A dense population of unmarked cells was observed in all nodule extracts due to contamination from plant material. Therefore, we decided that the J23104 promoter would be best to use for flow cytometry experiments, as this seems to label all cells. The Fix^- and Fix^{int} derivatives of Rlv3841 were already marked with GFP or mCherry with a J23104 promoter as in **Chapter 3**[108], and so these strains were ready to use in competition assays.

4.4.4 Assessment of fluorescently marked strains in competition using flow cytometry

As we chose the GFP and mCherry fluorescent proteins to label strains for flow cytometry competition assays, we needed to ensure that neither protein affected other aspects of rhizobial fitness, which might confound our interpretation of competition experiments. In **Chapter 3** we showed that these fluorescent proteins did not affect the growth rate (**Figure 3.7**), nitrogen fixation rate (**Figure 3.8**), nodulation ability (**3.4.4.5**), or the number of CFUs inside each nodule (**Figure 3.9**). Here, we tested whether the fluorescent proteins affected the number of bacteria or bacteroids within the nodule detected by the flow cytometer.

Plants were co-inoculated with a 1:1 ratio of GFP-marked Fix^+ (OPS1339) and mCherry-marked Fix^+ (OPS1341). One pre-germinated pea seed was potted per 500 ml pot

containing a 1:1 mixture of silver sand and fine vermiculite and co-inoculated with a 1:1 mixture of strains totalling 1 x 10^4 cells per pot. In two independent experiments, five replicate plants and one water control were grown for 28 days in a fully randomised design. At harvest, the ten largest nodules per strain per plant were selected for rhizobia extraction and flow cytometry, to ensure that all nodules were at a similar developmental stage. The number of cells was analysed separately for each cell type (bacteria or bacteroids), with a linear model as a function of fluorescent protein (GFP or mCherry). We attempted to include a random effect of plant to account for the fact that both GFP- and mCherry-marked nodules were sampled from the same plant, but the model would not converge.

In double inoculations, the absolute number of bacteria or bacteroids per nodule did not vary between the GFP- and mCherry-marked strains (bacteria $F_{1,18} = 0.2569$, $p = 0.6184$; bacteroids $F_{1,18} = 2.2194$, $p = 0.1536$; **Figure 4.5**). This demonstrates that the GFP and mCherry fluorescent proteins did not affect the number of bacteria or bacteroids within a nodule.

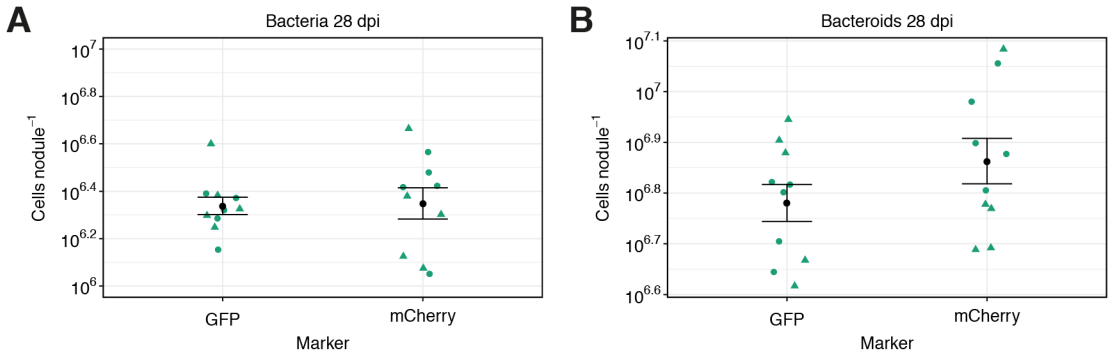


Figure 4.5 – The effect of fluorescent protein marker on the number of bacteria and bacteroids per nodule obtained from flow cytometry. The absolute number of (A) bacteria or (B) bacteroids per nodule did not vary between GFP- and mCherry-marked strains. (A, B) Points represent an average of three technical replicates per strain per independent replicate plant, with each technical replicate consisting of ten combined nodules. Circles and triangles indicate data from independent experiments. Black points indicate the overall mean with one standard error. Plants were grown for 28 days, n = 10. Values for group means and SEs are in **Table A1.1**.

4.4.5 Testing the effects of sanctioning on bacteria and bacteroids of Fix$^-$ using flow cytometry

To test how sanctioning affects the numbers of both bacteria and bacteroids within nodules, we inoculated plants with Fix$^+$ and Fix$^-$ in single and co-inoculations. Strains were marked with either GFP or mCherry so their nodules could be distinguished. The number of bacteria and bacteroids were quantified by flow cytometry and compared between treatments.

Plants were co-inoculated with a 1:1 ratio of GFP-marked Fix$^+$ (OPS1339) and mCherry-marked Fix$^-$ (OPS2271); or the mCherry-marked Fix$^+$ (OPS1341) and GFP-marked Fix$^-$ (OPS2270); or singly inoculated with one of each of these strains. One pre-germinated pea seed was potted per 1 L pot containing a 1:1 mixture of silver sand and fine vermiculite, and inoculated with either a single strain or a 1:1 mixture of strains totalling 1 x 10^4 cells per pot. In two independent experiments, two replicate plants per treatment per time point and two water controls per timepoint were grown for 28 or 56 days in a fully randomised design. At harvest, the ten largest nodules per strain per plant were selected for rhizobia extraction and flow cytometry, to ensure that all nodules were at a similar developmental stage.

The number of cells per nodule was analysed separately for each time point (28 and 56 dpi) and cell type (bacteria or bacteroids), with a linear model as a function of the identity of the strain (Fix$^+$ or Fix$^-$), the inoculation type (single or co-inoculation), and their interaction. For co-inoculated plants, both Fix$^+$ and Fix$^-$ nodules were sampled from the same plant. We attempted to include a random effect of plant to account for this, but the model would not converge.

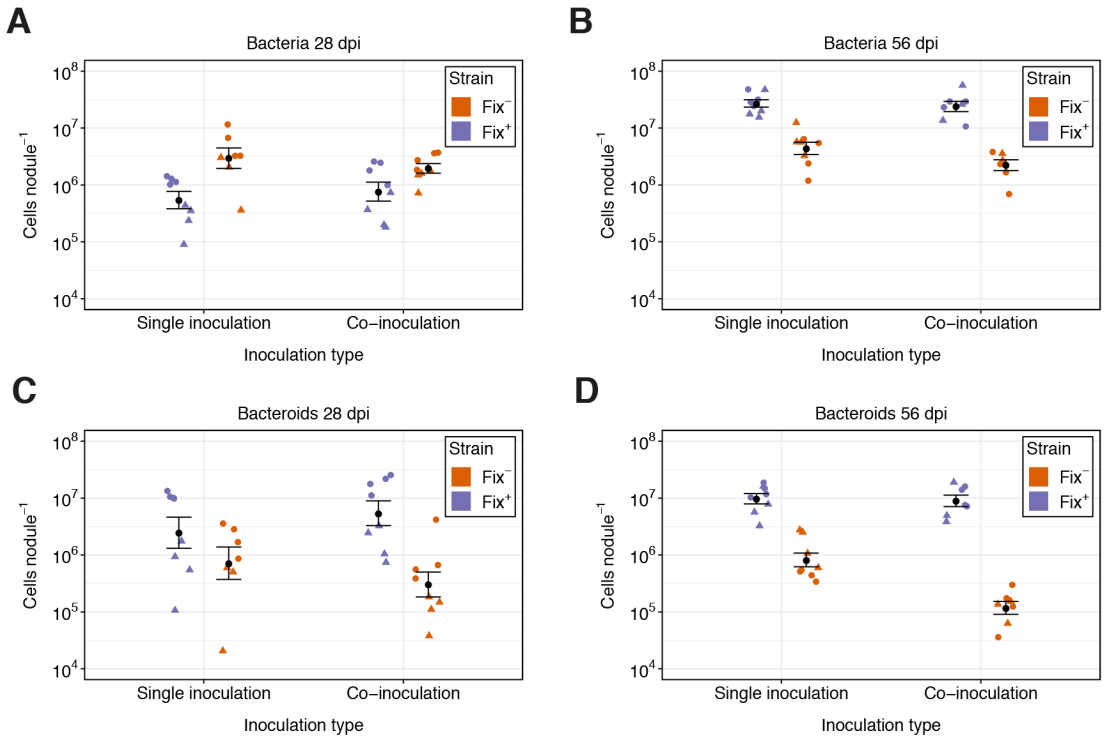


Figure 4.6 – Number of bacteria and bacteroids per nodule for Fix$^+$ and Fix$^-$ obtained from flow cytometry. (A) At 28 dpi Fix$^-$ nodules had more bacteria than Fix$^+$ nodules for both inoculation types. (B) At 56 dpi Fix$^-$ nodules had fewer bacteria than Fix$^+$ nodules for both inoculation types. (C) At 28 and (D) 56 dpi Fix$^-$ nodules had fewer bacteroids than Fix$^+$ nodules for both inoculation types. (A-D) Points represent an average of three technical replicates per strain per independent replicate plant, with each technical replicate consisting of ten combined nodules. Circles and triangles indicate data from independent experiments. Black points indicate the overall mean with one standard error, n = 7-8. Plants were grown for (A, C) 28 or (B, D) 56 days. Values for group means and SEs are in **Table A1.2**.

Cell type	ANOVA, interaction between inoculation type and strain	ANOVA, effect of inoculation type	ANOVA, effect of strain	Fix$^+$ and Fix$^-$ difference [95% CI]
Bacteria, 28 dpi	$F_{1,27} = 3.3835$, $p = 0.0769$	$F_{1,28} = 1.2827$, $p = 0.2670$	$F_{1,29} = 9.3123$, $p = 0.0048$	-2.2528 x 10^6 [-3.7626 x 10^6, -7.4294 x 10^5]*
Bacteria, 56 dpi	$F_{1,26} = 0.0084$, $p = 0.9277$	$F_{1,27} = 0.5089$, $p = 0.4817$	$F_{1,28} = 47.986$, $p < 0.0001$	2.4259 x 10^7 [1.7085 x 10^7, 3.1432 x 10^7]*
Bacteroids, 28 dpi	$F_{1,27} = 1.5212$, $p = 0.2281$	$F_{1,28} = 0.9264$, $p = 0.3440$	$F_{1,29} = 10.75$, $p = 0.0027$	7.1905 x 10^6 [2.7051 x 10^6, 1.1676 x 10^7]*
Bacteroids, 56 dpi	$F_{1,26} = 0.0062$, $p = 0.9288$	$F_{1,27} = 0.3374$, $p = 0.5661$	$F_{1,28} = 50.392$, $p < 0.0001$	1.0167 x 10^7 [7.2331 x 10^6, 1.3101 x 10^7]*

Table 4.2 - Linear model results for the number of bacteria or bacteroids in nodules when Fix$^+$ and Fix$^-$ were applied in single inoculations or co-inoculations. * represent where 95% CIs indicate significant differences between groups.

In all cases the interaction between inoculation type and strain was not significant (**Table 4.2**). This shows that, for all cell types, the difference between the number of Fix^+ and Fix^- cell numbers did not depend on whether the strain is co-inoculated with another strain, or is the only strain on the plant. As the interaction terms were not significant, these were removed from all models, leaving inoculation type and strain as additive effects. Here, in all cases the effect of inoculation type was not significant (**Table 4.2**). This shows that the number of cells did not depend on whether the strain is singly or co-inoculated.

As the effect of inoculation type was not significant, this term was removed from all models. In all cases, the number of cells per nodule did depend on whether the strain was Fix^+ or $Fix^–$, as strain had a significant effect on the fit of all models (**Table 4.2**). $Fix^–$ had more bacteria per nodule than Fix^+ at 28 dpi (**Figure 4.6*A***), but fewer at 56 dpi (**Figure 4.6*B***). Contrastingly, $Fix^–$ had fewer bacteroids per nodule at both time points (**Figure 4.6*C, D***).

These results demonstrate that by 56 dpi, $Fix^–$ nodules had fewer bacteria and bacteroids than Fix^+ nodules. However, we cannot state that this effect was due to conditional sanctioning of $Fix^–$ in response to other available strains, rather than a general response to $Fix^–$ strains, as the number of cells did not depend on whether the strain was singly or co-inoculated.

4.4.6 Testing the effects of sanctioning on bacteria and bacteroids of Fix^{int} using flow cytometry

Given the evidence of reduced bacteria and bacteroid numbers for Fix^{-} nodules, we then tested how bacteria and bacteroid numbers are affected when a Fix^{int} strain is sanctioned[108]. Plants were co-inoculated with Fix^{int} in competition with Fix^{+}, Fix^{-}, or a differently-marked version of itself. Strains were marked with either GFP or mCherry so their nodules could be distinguished. The number of bacteria and bacteroids in Fix^{int} nodules was quantified by flow cytometry and compared between treatments.

Plants were co-inoculated with a 1:1 ratio of GFP-marked Fix^{int} (OPS2268) and mCherry-marked Fix^{+} (OPS1341), Fix^{int} (OPS2269), or Fix^{-} (OPS2271); or the mCherry-marked Fix^{int} (OPS2269) and GFP-marked Fix^{+} (OPS1339), Fix^{int} (OPS2268), or Fix^{-} (OPS2270). One pre-germinated pea seed was potted per 1 L pot containing a 1:1 mixture of silver sand and fine vermiculite and co-inoculated with a 1:1 mixture of strains totalling 1 x 10^4 cells per pot. In three independent experiments, one replicate plant per treatment per time point and two water controls per timepoint were grown for 28 or 56 days in a fully randomised design. At harvest, the ten largest nodules per plant were selected for rhizobia extraction and flow cytometry, to ensure that all nodules were at a similar developmental stage.

The number of cells per nodule was analysed separately for each time point (28 and 56 dpi) and cell type (bacteria and bacteroids), in a linear model as a function of the competing strain (Fix^{+}, Fix^{int}, Fix^{-}). As we had two degrees of freedom, we could make comparisons between two pairs of data. We compared the number of Fix^{int} cells when competing against Fix^{+} and Fix^{int}, and against Fix^{+} and Fix^{-}, as in both cases we expected

Fix^{int} nodules to have fewer cells when competing against Fix^{+} if sanctioning occurs. We did not have a hypothesis about whether the number of Fix^{int} cells would be different when competing against Fix^{int} or Fix^{-}, so this comparison was not made.

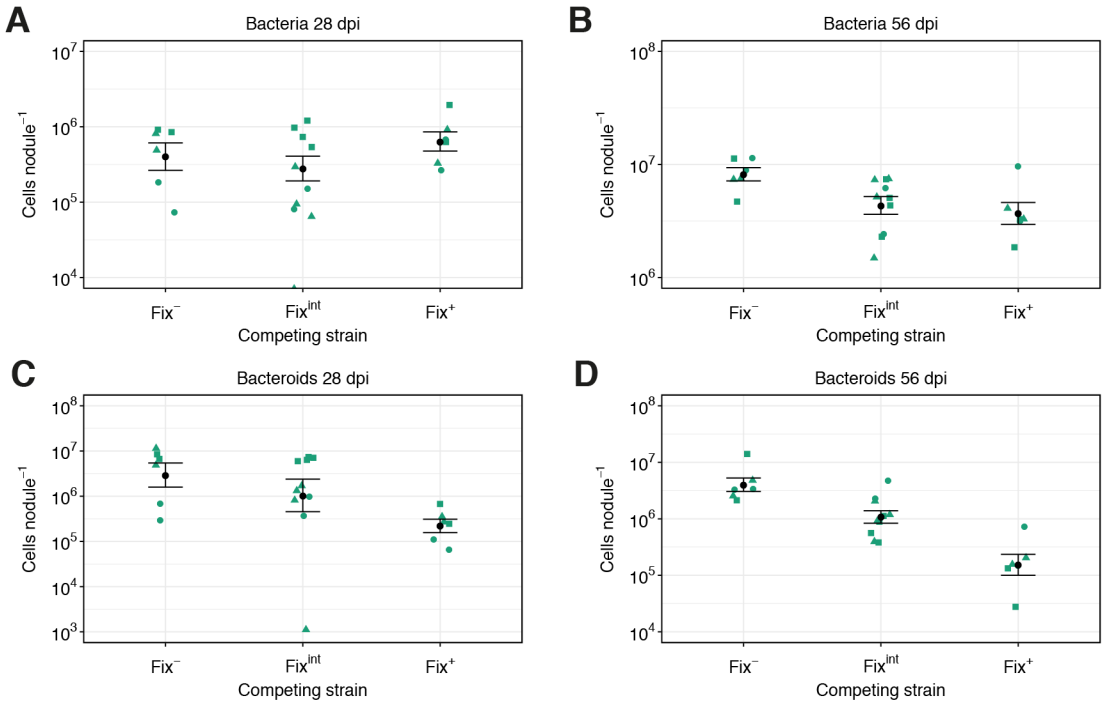


Figure 4.7 – Number of bacteria and bacteroids per nodule for Fix^{int} obtained from flow cytometry. (A) At 28 dpi Fix^{int} had similar numbers of bacteria per nodule when competing against Fix^{+}, itself, or Fix^{-}. (B) At 56 dpi Fix^{int} had significantly more bacteria per nodule when competing against Fix^{-}, compared to against Fix^{+}. At (C) 28 and (D) 56 dpi Fix^{int} had significantly more bacteroids per nodule when competing against Fix^{-}, compared to against Fix^{+}. (A-D) Points represent an average of three technical replicates per strain per independent replicate plant, with each technical replicate consisting of ten combined nodules. Circles, triangles, and squares indicate data from independent experiments. Black points indicate the overall mean with one standard error, n = 5-6. Plants were grown for (A, C) 28 or (B, D) 56 days. Values for group means and SEs are in **Table A1.3.**

Cell type	ANOVA, effect of competing strain	Competing strains compared	Difference in Fix^{int} cells when competing against Fix^{+} and against Fix^{int}/Fix^{-} [95 % CI]
Bacteria, 28 dpi	$F_{2,19} = 1.2419$, $p = 0.3112$	Fix^{+} and Fix^{int}	3.8200 x 10^5 [-3.2476 x 10^5, 8.0965 x 10^5]
		Fix^{+} and Fix^{-}	2.4245 x 10^5 [-1.2534 x 10^5, 8.8931 x 10^5]
Bacteria, 56 dpi	$F_{2,19} = 5.4497$, $p = 0.0135$	Fix^{+} and Fix^{int}	-0.6896 x 10^6 [-3.3852 x 10^6, 2.0062 x 10^6]
		Fix^{+} and Fix^{-}	-4.3205 x 10^6 [-7.3343 x 10^6, -1.3066 x 10^6]*
Bacteroids, 28 dpi	$F_{2,19} = 4.1678$, $p = 0.0316$	Fix^{+} and Fix^{int}	-2.9028 x 10^6 [-6.2321 x 10^6, 0.4266 x 10^6]
		Fix^{+} and Fix^{-}	-5.1134 x 10^6 [-8.8358 x 10^6, -1.3911 x 10^6]*
Bacteroids, 56 dpi	$F_{2,19} = 0.0316$, $p = 0.0084$	Fix^{+} and Fix^{int}	-1.2212 x 10^6 [-3.9130 x 10^6, 1.4707 x 10^6]
		Fix^{+} and Fix^{-}	-4.8133 x 10^6 [-7.8228 x 10^6, -1.8037 x 10^6]*

Table 4.3 - Linear model results for the number of bacteria or bacteroids in Fix^{int} nodules when competing against Fix^{+}, itself or Fix^{-}. * represent where 95% CIs indicate significant differences between groups.

Sanctioning had different effects on the number of Fix^{int} bacteria at 28 and 56 dpi. The number of bacteria per Fix^{int} nodule was not dependent on the competing strain at 28 dpi (**Figure 4.7*A*, Table 4.3**). But this had changed by 56 dpi, as Fix^{int} had significantly more bacteria per nodule when competing against Fix^{-} compared to against Fix^{+} (**Figure 4.7*B*, Table 4.3**).

The number of bacteroids per Fix^{int} nodule was dependent on the competing strain at both 28 and 56 dpi (**Figure 4.7*C*, *D*, Table 4.3**). At 28 and 56 dpi, Fix^{int} had significantly more bacteroids per nodule when competing against Fix^{-} compared to against Fix^{+} (**Figure 4.7*C*, *D*, Table 4.3**).

Together, these results demonstrate that sanctioning of Fix^{int} nodules results in fewer bacteria and bacteroids per nodule when Fix^{int} is co-inoculated with a better fixer. The effects of sanctioning can be seen at different times for the different populations of rhizobia within a nodule: by 28 dpi for bacteroids and by 56 dpi for undifferentiated bacteria.

4.4.7 Testing the effects of sanctioning within mixed nodules

To test whether plants can sanction non-fixers within mixed nodules, and how this affects the numbers of bacteria and bacteroids, we compared cell numbers in nodules containing one Fix^{+} and one Fix^{-} strain. Mixed nodules containing either two differently-marked Fix^{+} strains, or two differently-marked Fix^{-} strains were used as a comparison, to see if strains did better or worse in a mixed nodule when cohabiting with a strain with the same fixation rate, or a different fixation rate. Strains were marked with either GFP or mCherry so that mixed nodules could be identified using the fluorescence microscope, and the strains

inside distinguished within the flow cytometer. The number of bacteroids and bacteria were quantified by flow cytometry and compared between treatments. Additionally, to compare our results to those of Daubech *et al.* (2017)[107], the number of CFUs of each strain in the nodule was also measured by drop plate counts.

Plants were co-inoculated with a 1:1 ratio of GFP-marked Fix^+ (OPS1339) and mCherry-marked Fix^+ (OPS1341); GFP-marked Fix^- (OPS2270) and mCherry-marked Fix^- (OPS2271); or GFP-marked Fix^+ (OPS1339) and mCherry-marked Fix^- (OPS2271). One pre-germinated pea seed was potted per 1 L pot containing a 1:1 mixture of silver sand and fine vermiculite and co-inoculated with a 1:1 mixture of strains totalling 1 x 10^8 cells per pot. In three independent experiments, four replicate plants per treatment per time point and two water controls per timepoint were grown for 28 or 56 days in a fully randomised design. At harvest, one mixed nodule per plant was selected for imaging, rhizobia extraction, flow cytometry, serial dilutions and drop plate counts. Where more than one mixed nodule could be identified, the largest mixed nodule per plant was selected.

The number of cells per nodule was analysed separately for each time point (28 and 56 dpi) and cell type (CFUs, bacteria, and bacteroids), with a linear model as a function of the identity of the strain (Fix^+ or Fix^-), the mixed nodule occupants (whether the cohabiting strain had the same or a different fixation ability) and their interaction. We attempted to include a random effect of plant to account for the fact that two strains were measured from within the same nodule, but the model would not converge. The percentage of cells which were Fix^- was analysed separately for each time point (28 and 56 dpi) and cell type (CFUs, bacteria, and bacteroids), in a generalised linear model as a

function of the identity of the mixed nodule occupants (whether the cohabiting strain had the same or a different fixation ability) using a quasibinomial error distribution.

For the number of CFUs from drop plate counts, at both time points the interaction between mixed nodule occupants and strain was not significant (**Table 4.4**). This shows that the difference between the number of Fix^+ and Fix^- CFUs in mixed nodules did not depend on whether the cohabiting strain had the same or a different fixation rate. As the interaction term was not significant, this was removed from both models, leaving mixed nodule occupants and strain as additive effects. Again, at both time points the effect of the mixed nodule occupants was not significant (**Table 4.4**), so this term was removed from both models. This shows that the number of CFUs did not depend on whether the strain cohabits a nodule with a strain that has the same fixation rate, or a different fixation rate. At 28 dpi, Fix^- strains had more CFUs than Fix^+ strains in mixed nodules (**Figure 4.8*A*, Table 4.4**), but at 56 dpi both strains had the same number of CFUs (**Figure 4.8*B*, Table 4.4**), which does not support the hypothesis that plants can sanction within mixed nodules.

Given the large variability in absolute CFUs number, the percentage of CFUs per mixed nodule was also compared. Here, Fix^- formed a greater percentage of the CFUs at 28 dpi when cohabiting with Fix^+ than with a differently-marked version of itself (**Figure 4.8*C*, Table 4.4**), but there was no significant difference at 56 dpi (**Figure 4.8*D*, Table 4.4**), which again does not support the hypothesis that plants can sanction within mixed nodules.

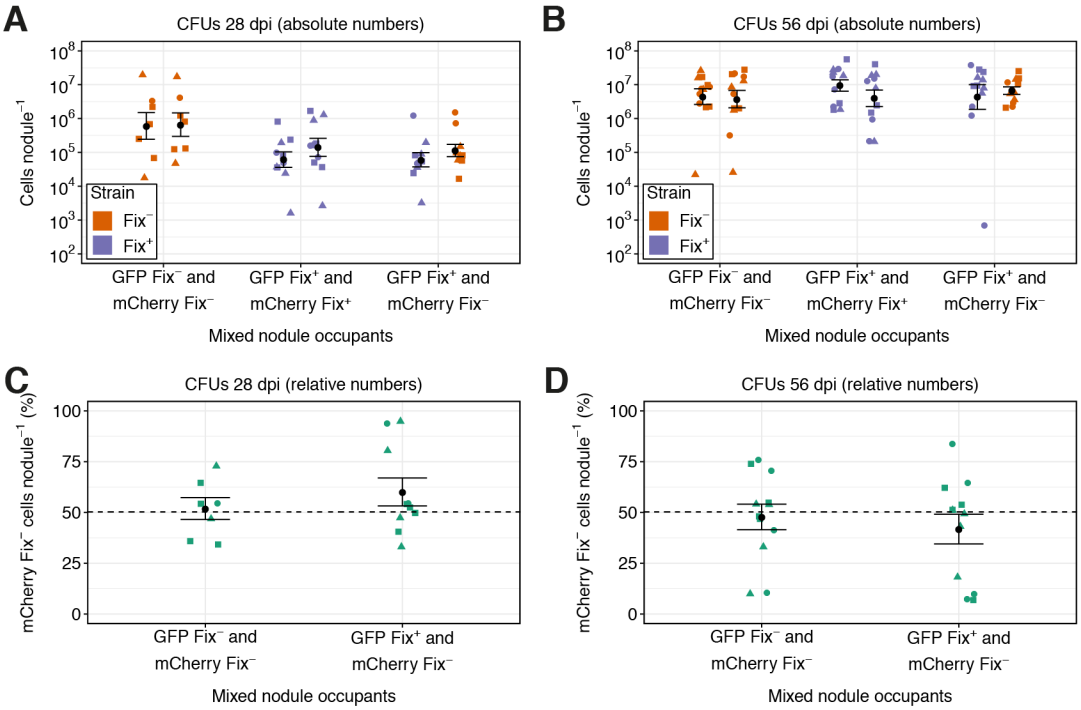


Figure 4.8 – Number of CFUs per mixed nodule for Fix$^+$ and Fix$^-$ obtained from drop plate counts. The number of Fix$^+$ or Fix$^-$ CFUs per mixed nodule at (A) 28 and (B) 56 dpi did not depend on the cohabiting strain. (A, B) The left-hand points for each pair represent the GFP-marked strain, and the right-hand the mCherry marked strain. (C, D) Dashed line represents where 50% of CFUs are mCherry-marked Fix$^-$. (A-D) Points represent an average of five technical replicates per strain per independent replicate plant, with each replicate plant consisting of a single nodule. Circles, triangles, and squares indicate data from independent experiments. Black points indicate the overall mean with one standard error. (A, C) Plants were grown for 28 days, n = 7-10. (B, D) Plants were grown for 56 days, n = 11-12. Values for group means and SEs are in **Table A1.4**.

Cell type	**ANOVA, interaction between cohabiting strain and strain**	**ANOVA, effect of cohabiting strain**	**ANOVA, effect of strain**	**Fix$^+$ and Fix$^-$ difference [95 % CI]**
CFUs count, 28 dpi	$F_{1,50} = 2.8516$, $p = 0.0975$	$F_{1,51} = 2.8115$, $p = 0.0997$	$F_{1,52} = 4.2744$, $p = 0.0437$	-1.9334 x 10^6 [-3.8099 x 10^6, -5.6877 x 10^4]*
CFUs count, 56 dpi	$F_{1,66} < 0.0001$, $p = 0.9975$	$F_{1,67} = 0.1461$, $p = 0.7035$	$F_{1,68} = 3.0508$, $p = 0.0852$	4.6313 x 10^6 [-6.5976 x 10^5, 9.9223 x 10^6]
Cell type	**ANOVA, effect of cohabiting strain**			**Difference when cohabiting with Fix$^+$ or Fix$^-$ [95 % CI]**
Fix$^-$ CFUs (%), 28 dpi	$F_{1,15} = 4.4132$, $p = 0.0530$			OR 1.71010 [1.0305, 2.8407]*
Fix$^-$ CFUs (%), 56 dpi	$F_{1,15} = 2.1771$, $p = 0.1542$			OR 0.5638 [0.2624, 1.2114]

Table 4.4 - Linear and generalised linear model results for the number or percentage of Fix$^+$ or Fix$^-$ CFUs per nodule when in mixed nodules. * represent where 95% CIs indicate significant differences between groups.

Comparably to the drop plate count data, the flow cytometry data showed that for bacteria and bacteroids at both time points the interaction between mixed nodule occupants and strain was not significant (**Table 4.5**), so this term was removed from all models. Similarly, for bacteria and bacteroids, at both time points the effect of mixed nodule occupants was not significant (**Table 4.5**), so this term was removed from both models. This shows that the number of bacteria or bacteroids did not depend on whether the strain cohabits a nodule with a strain that has the same fixation rate, or a different fixation rate. At 28 dpi, Fix^- strains had more bacteria than Fix^+ strains per mixed nodule (**Figure 4.9*A***, **Table 4.5**) but fewer at 56 dpi (**Figure 4.9*B***, **Table 4.5**). At both 28 dpi and 56 dpi Fix^- strains had fewer bacteroids than Fix^+ strains (**Figure 4.9*C, D***, **Table 4.5**). However, we cannot state that this effect was due to conditional within-nodule sanctioning of Fix^- in response to other available strains, rather than a general response to Fix^- strains, as the number of cells did not depend on whether the strain cohabited a nodule with a strain that had the same, or a different, fixation rate.

Given the large variation in the absolute numbers of bacteria and bacteroids per nodule, we also compared the percentage of bacteria or bacteroids which were mCherry-marked Fix^-, when sharing a mixed nodule with the GFP-marked Fix^- or Fix^+. At 28 dpi, Fix^- formed a greater percentage of bacteria per mixed nodule when sharing with Fix^+, as opposed to a differently-marked version of itself (**Figure 4.9*E***, **Table 4.5**). At 56 dpi, Fix^- formed a smaller percentage of bacteroids per mixed nodule when sharing with Fix^+, as opposed to a differently-marked version of itself (**Figure 4.9*H***, **Table 4.5**), which shows that sanctioning within a mixed nodule can reduce the number of bacteroids.

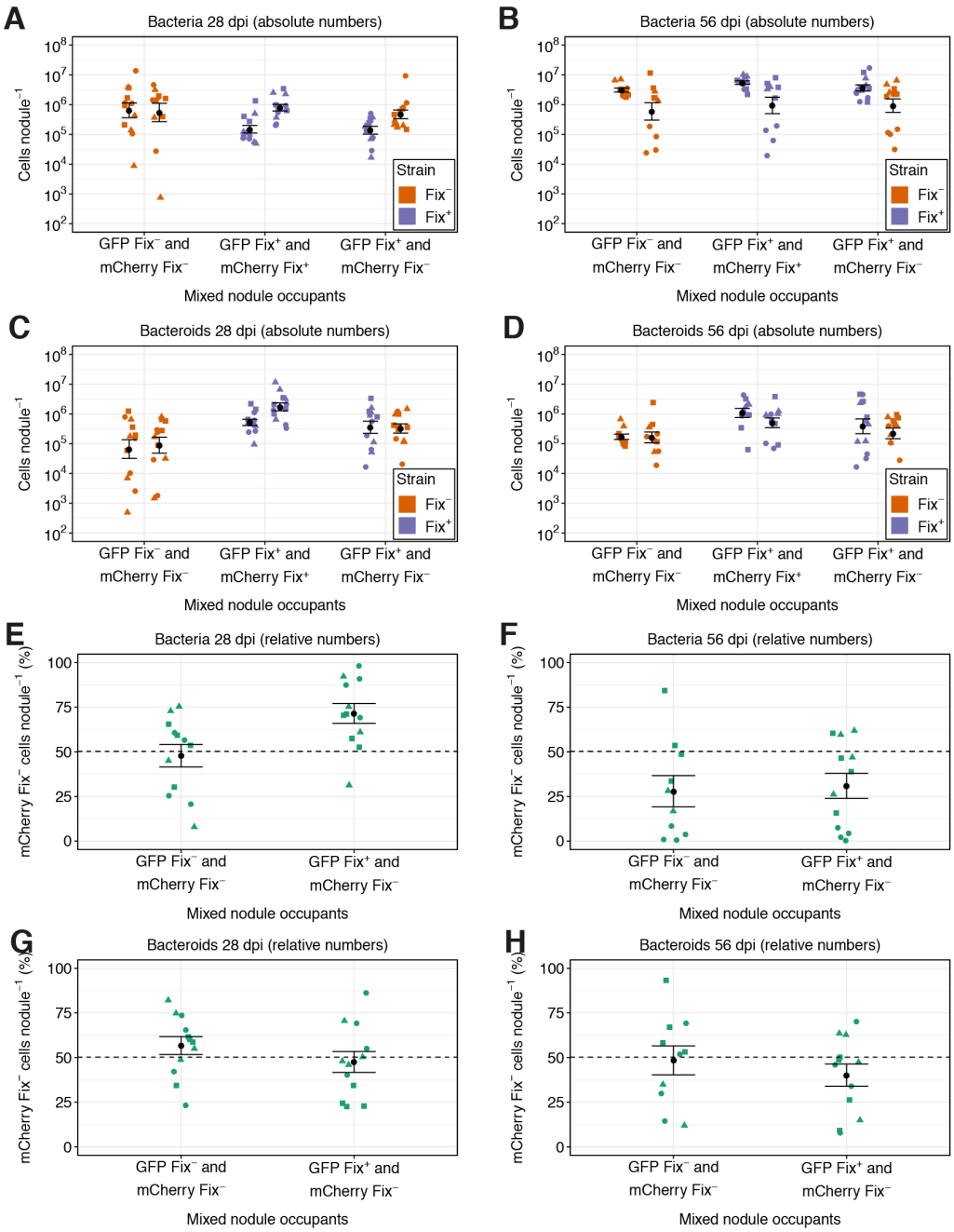


Figure 4.9 – Number of bacteria and bacteroids per mixed nodule for Fix$^+$ and Fix$^-$ obtained from flow cytometry. The number of Fix$^+$ or Fix$^-$ bacteria per mixed nodule at (A) 28 and (B) 56 dpi, or bacteroids at (C) 28 and (D) 56 dpi did not depend on the cohabiting strain. (A, B) The left-hand points for each pair represent the GFP-marked strain, and the right-hand the mCherry marked strain. (E) At 28 dpi Fix$^-$ formed a larger percentage of bacteria per mixed nodule when sharing a nodule with Fix$^+$. (F) At 56 dpi the percentage of bacteria per mixed nodule which were Fix$^-$ did not depend on the cohabiting strain. (G) At 28 dpi the percentage of bacteroids per mixed nodule which were Fix$^-$ did not depend on the cohabiting strain. (H) At 56 dpi Fix$^-$ formed a smaller percentage of bacteroids per mixed nodule when sharing a nodule with Fix$^+$. (A-H) Points represent an average of three technical replicates per strain per independent replicate plant, with each replicate plant consisting of a single nodule. Circles, triangles, and squares indicate data from independent experiments. Black points indicate the overall mean with one standard error. (E-H) Dashed line represents where 50% of cells are mCherry-marked Fix$^-$. (A, C, E, G) Plants were grown for 28 days, n = 12. (B, D, F, H) Plants were grown for 56 days, n = 10-12. Values for group means and SEs are in **Table A1.4**.

Cell type	ANOVA, interaction between cohabiting strain and strain	ANOVA, effect of cohabiting strain	ANOVA, effect of strain	Fix^+ and Fix^- difference [95 % CI]
Bacteria count, 28 dpi	$F_{1,68} = 0.0340$, $p = 0.8543$	$F_{1,69} = 1.3291$, p 0.2529	$F_{1,70} = 5.552$, $p = 0.02126$	-1.1378×10^6 [-2.1009×10^6, -1.7472×10^5]*
Bacteria count, 56 dpi	$F_{1,62} = 0.6850$, $p = 0.4110$	$F_{1,63} = 0.0032$, $p = 0.9551$	$F_{1,64} = 6.6114$, $p = 0.0125$	2.0335×10^6 [4.5359×10^5, 3.6135×10^6]*
Bacteroid count, 28 dpi	$F_{1,68} = 2.4190$, $p = 0.1245$	$F_{1,69} = 0.7729$, $p = 0.3824$	$F_{1,70} = 8.8338$, $p = 0.0041$	1.0977×10^6 [3.6110×10^5, 1.8343×10^6]*
Bacteroid count, 56 dpi	$F_{1,62} < 0.0001$, $p = 0.9970$	$F_{1,63} = 0.1145$, $p = 0.7362$	$F_{1,64} = 13.851$, $p = 0.0004$	9.6058×10^5 [4.4496×10^5, 1.4762×10^6]*
Cell type	**ANOVA, effect of cohabiting strain**			**Difference when cohabiting with Fix^+ or with Fix^- [95 % CI]**
Fix^- bacteria (%), 28 dpi	$F_{1,22} = 27.268$, $p < 0.0001$			OR 8.8676 [3.4416, 22.8486]*
Fix^- bacteria (%), 56 dpi	$F_{1,20} = 0.6914$, $p = 0.4155$			OR 0.6505 [0.2362, 1.7910]
Fix^- bacteroid (%), 28 dpi	$F_{1,22} = 0.6756$, $p = 0.4199$			OR 0.7557 [0.3879, 1.4724]
Fix^- bacteroid (%), 56 dpi	$F_{1,20} = 11.687$, $p = 0.0027$			OR 0.1585 [0.0532, 0.4725]*

Table 4.5 - Linear and generalised linear model results for the number or percentage of Fix^+ or Fix^- bacteria or bacteroids per nodule when in mixed nodules. * represent where 95% CIs indicate significant differences between groups.

Together, these results show limited evidence of sanctioning within mixed nodules. The percentage of bacteroids which were Fix^- was lower when cohabiting a mixed nodule with Fix^+ at 56 dpi. Additionally, by 56 dpi and Fix^- strains had fewer bacteria and bacteroids per mixed nodule than Fix^+ strains. However, we cannot state that this effect was due to conditional sanctioning of Fix^- in response to other available strains, rather than a general response to Fix^- strains, as the number of CFUs, bacteria, and bacteroids, did not depend on whether the strain was singly or co-inoculated.

4.4.8 Testing the effects of sanctioning on an intermediate fixer within mixed nodules

Despite the limited evidence for sanctioning of a non-fixer within mixed nodules, we also assessed whether plants can sanction an intermediate fixer within mixed nodules. We compared cell numbers in mixed nodules that contained a Fix^{int} strain in combination with Fix^{+}, Fix^{-}, or a differently-marked version of itself. Strains were marked with either GFP or mCherry so that mixed nodules could be identified using the fluorescence microscope, and the strains inside distinguished within the flow cytometer. The number of bacteroids and bacteria were quantified by flow cytometry and compared between treatments. Additionally, to compare our results to those of Daubech *et al.* (2017)[107], we conducted drop plate counts to compare the number of CFUs per strain.

Plants were co-inoculated with a 1:1 ratio of GFP-marked Fix^{int} (OPS2268) and mCherry-marked Fix^{+} (OPS1341), Fix^{int} (OPS2269), or Fix^{-} (OPS2271). One pre-germinated pea seed was potted per 1 L pot containing a 1:1 mixture of silver sand and fine vermiculite and co-inoculated with a 1:1 mixture of strains totalling 1 x 10^8 cells per pot. In three independent experiments, four replicate plants per treatment per time point and two water controls per timepoint were grown for 28 or 56 days in a fully randomised design. At harvest, one mixed nodule per plant was selected for imaging, rhizobia extraction, flow cytometry, serial dilutions and drop plate counts. Where more than one mixed nodule could be identified, the largest mixed nodule per plant was selected.

The number of Fix^{int} cells per mixed nodule was analysed separately for each cell type (CFUs, bacteria and bacteroids) at each time point (28 and 56 dpi), in a linear model as a function of the cohabiting strain (Fix^{+}, Fix^{int}, Fix^{-}). As we had two degrees of freedom,

we could make comparisons between two pairs of data. We compared the number of Fix^{int} cells when sharing a nodule with Fix^{+} or Fix^{int}; and with Fix^{+} or Fix^{-}, as in both cases we expected Fix^{int} to have fewer cells when sharing a nodule with Fix^{+} if within-nodule sanctioning occurs. We did not have a hypothesis about whether the number of Fix^{int} cells would be different when sharing with Fix^{int} or Fix^{-}, so this comparison was not made. The percentage of cells which were Fix^{int} was also analysed separately for each cell type at each time point, in a generalised linear model as a function of the cohabiting strain using a quasibinomial error distribution.

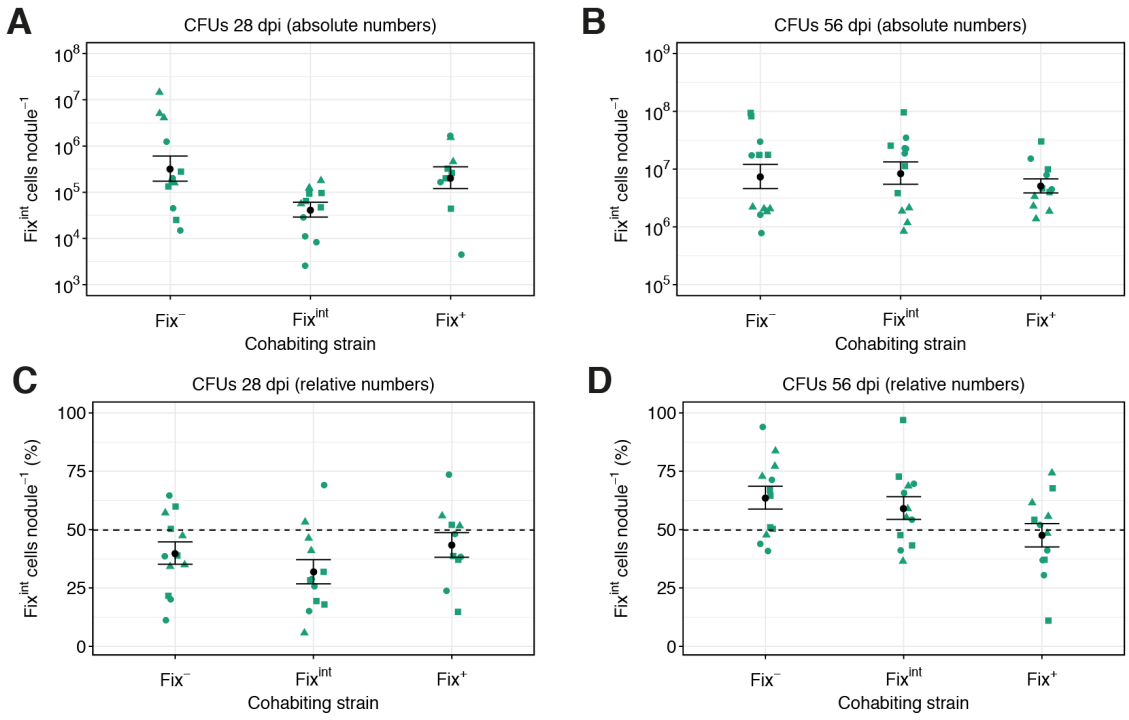


Figure 4.10 – Number of CFUs per mixed nodule for Fix^{int} obtained from drop plate counts. (A) At 28 and (B) 56 dpi Fix^{int} had similar numbers of CFUs per mixed nodule when cohabiting with Fix^{+}, itself, or Fix^{-}. (C) At 28 dpi, the percentage of CFUs per mixed nodule which were Fix^{int} was larger when cohabiting with Fix^{+}, compared to with itself. (D) At 56 dpi, the percentage of CFUs per mixed nodule which were Fix^{int} was similar when cohabiting with Fix^{+}, itself, or Fix^{-}. (A-D) Points represent an average of five technical replicates per strain per independent replicate plant, with each replicate plant consisting of a single nodule. Circles, triangles, and squares indicate data from independent experiments. Black points indicate the overall mean with one standard error. (C, D) Dashed line represents where 50% of CFUs are GFP-marked Fix^{int}. (A, C) Plants were grown for 28 days, n = 10-12. (B, D) Plants were grown for 56 days, n = 12. Values for group means and SEs are in **Table A1.5**.

Cell type	ANOVA, effect of cohabiting strain	Cohabiting strains compared	Difference in Fix^{int} cells when cohabiting with Fix^{+} and with Fix^{int}/Fix^{-} [95 % CI]
CFU count, 28 dpi	$F_{2,31}$ = 2.2327, p = 0.1242	Fix^{+} and Fix^{int}	4.2105 x 10^5 [-1.8026 x 10^6, 2.6447 x 10^6]
		Fix^{+} and Fix^{-}	-1.6688 x 10^6 [-3.8924 x 10^6, 0.5548 x 10^6]
CFU count, 56 dpi	$F_{2,33}$ = 1.3679, p = 0.2687	Fix^{+} and Fix^{int}	-1.3135 x 10^7 [-3.3600 x 10^7, 0.7330 x 10^7]
		Fix^{+} and Fix^{-}	-1.5411 x 10^7 [-3.5875 x 10^7, 0.5054 x 10^7]
CFU (%), 28 dpi	$F_{2,31}$ = 3.7501, p = 0.0348	Fix^{+} and Fix^{int}	OR 0.5715 [0.3481, 0.9383]*
		Fix^{+} and Fix^{-}	OR 1.0954 [0.6834, 1.7557]
CFU (%), 56 dpi	$F_{2,35}$ = 2.0006, p = 0.1513	Fix^{+} and Fix^{int}	OR 1.5182 [0.8614, 2.6760]
		Fix^{+} and Fix^{-}	OR 1.7633530 [0.9844, 3.1587]

Table 4.6 – Linear and generalised linear model results for the number or percentage of Fix^{int} CFU per nodule when in mixed nodules with Fix^{+}, itself or Fix^{-}. * represent where 95% CIs indicate significant differences between groups.

The drop plate data showed that the absolute number of Fix^{int} CFUs per mixed nodule was not dependent on the identity of the cohabiting strain at 28 or 56 dpi (**Figure 4.10*A***, ***B,*** **Table 4.6**). However, when expressed as a percentage of all CFUs, there was weak evidence that the identity of the cohabiting strain affected the relative success of the Fix^{int} strain in mixed nodules at 28 dpi (**Figure 4.10*C,*** **Table 4.6**) but not at 56 dpi (**Figure 4.10*D,*** **Table 4.6**). At 28 dpi the GFP-marked Fix^{int} appeared to be less successful in mixed nodules that were cohabited by the mCherry-marked Fix^{int} strain (**Figure 4.10*C,*** **Table 4.6**). However, there is no intuitive explanation for this, as **Figure 3.9** showed the fluorescent marker not to affect the number of CFUs per nodule.

For the flow cytometry data, the absolute numbers of Fix^{int} bacteria and bacteroids in mixed nodules did not depend on the identity of the cohabiting strain at 28 or 56 dpi (**Figure 4.11*A-D,*** **Table 4.7**). However, the percentage of bacteria which were Fix^{int} per mixed nodule was affected by the cohabiting strain at 56 dpi (**Figure 4.11*E,*** **Table 4.7**),

but not at 28 dpi (**Figure 4.11*F*, Table 4.7**). At 56 dpi, Fix^{int} formed a smaller percentage of the bacteria per nodule when cohabiting with Fix^{+} compared to when cohabiting with Fix$^{–}$ or a differently-marked Fix^{int} strain (**Figure 4.11*F*, Table 4.7**), which supports the hypothesis that plants can conditionally sanction poor fixers within mixed nodules.

For bacteroids, the percentage which were Fix^{int} within mixed nodules depended on the cohabiting strain at both 28 and 56 dpi (**Figure 4.11G**, ***H*, Table 4.7**). At 28 dpi, Fix^{int} formed a larger percentage of the bacteroids in each nodule when cohabiting with Fix$^{–}$ compared to when sharing with Fix^{+} or with another Fix^{int} strain (**Figure 4.11*G*, Table 4.7**). At 56 dpi, the percentage of bacteroids which were Fix^{int} was higher when cohabiting a nodule with Fix$^{–}$ or a differently-marked version of itself, compared to cohabiting with Fix^{+}, in line with predictions from within-nodule conditional sanctioning (**Figure 4.11*H*, Table 4.7**).

Cell type	ANOVA, effect of cohabiting strain	Cohabiting strains compared	Difference in Fix^{int} cells when cohabiting with Fix^{+} and with Fix^{int}/Fix$^{–}$ [95 % CI]
Bacteria count, 28 dpi	$F_{2,33}$ = 2.6199, p = 0.0879	Fix^{+} and Fix^{int}	0.7703 x 10^5 [-2.3166 x 10^5, 3.8571 x 10^5]
		Fix^{+} and Fix$^{–}$	-2.5477 x 10^5 [-5.6346 x 10^5, 0.5391 x 10^5]
Bacteria count, 56 dpi	$F_{2,33}$ = 0.2791, p = 0.7582	Fix^{+} and Fix^{int}	-0.5578 x 10^6 [-3.0428 x 10^6, 1.9271 x 10^6]
		Fix^{+} and Fix$^{–}$	-0.9043 x 10^6 [-3.3893 x 10^6, 1.5806 x 10^6]
Bacteroid count, 28 dpi	$F_{2,33}$ = 1.9237, p = 0.1621	Fix^{+} and Fix^{int}	3.7642 x 10^5 [-4.3902 x 10^4, 7.9674 x 10^5]
		Fix^{+} and Fix$^{–}$	0.5823 x 10^5 [-3.6209 x 10^5, 4.7855 x 10^5]
Bacteroid count, 56 dpi	$F_{2,33}$ = 1.9237, p = 0.2421	Fix^{+} and Fix^{int}	-1.4267 x 10^6 [-3.1209 x 10^6, 0.2676 x 10^6]
		Fix^{+} and Fix$^{–}$	-0.5937 x 10^6 [-2.2879 x 10^6, 1.1006 x 10^6]
Bacteria (%), 28 dpi	$F_{2,33}$ = 1.5008, p = 0.2378	Fix^{+} and Fix^{int}	OR 0.6625 [0.2156, 2.0357]
		Fix^{+} and Fix$^{–}$	OR 1.4400 [0.6977, 2.9720]
Bacteria (%), 56 dpi	$F_{2,33}$ = 4.4454, p = 0.0195	Fix^{+} and Fix^{int}	OR 2.2726 [1.0685, 4.8334]*
		Fix^{+} and Fix$^{–}$	OR 2.9386 [1.3509, 6.3922]*
Bacteroid (%), 28 dpi	$F_{2,33}$ = 4.8789, p = 0.0139	Fix^{+} and Fix^{int}	OR 0.4234 [0.1851, 0.9688]*
		Fix^{+} and Fix$^{–}$	OR 1.5823 [0.7803, 3.2089]
Bacteroid (%), 56 dpi	$F_{2,33}$ = 7.1472, p = 0.0026	Fix^{+} and Fix^{int}	OR 3.8705 [1.7192, 8.7139]*
		Fix^{+} and Fix$^{–}$	OR 4.2535 [1.6972, 10.6605]*

Table 4.7 – Linear model results for the number or percentage of Fix^{int} bacteria or bacteroids per nodule when in mixed nodules with Fix^{+}, itself or Fix$^{–}$. * represent where 95% CIs indicate significant differences between groups.

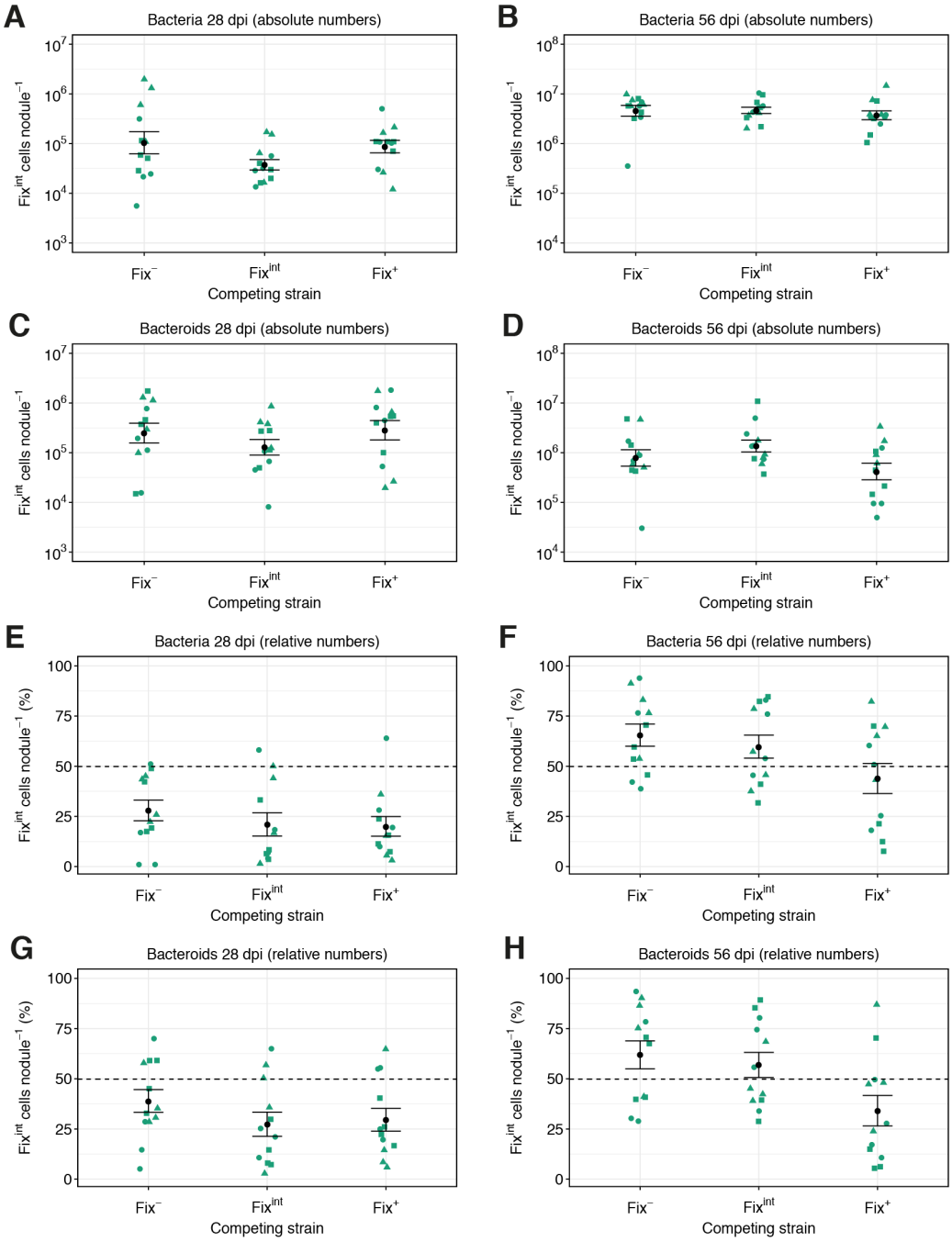


Figure 4.11 – Number of bacteria and bacteroids per mixed nodule for Fix^{int} obtained from flow cytometry. At 28 and 56 dpi Fix^{int} had similar numbers of (A, B) bacteria and (C, D) bacteroids per nodule when cohabiting with Fix^{+}, itself, or Fix^{-}. (E) At 28 dpi, the percentage of bacteria per mixed nodule which were Fix^{int} was similar when cohabiting with Fix^{+}, itself, or Fix^{-}. (F) At 56 dpi, the percentage of bacteria per mixed nodule which were Fix^{int} was smaller when cohabiting with Fix^{+}, compared to with itself, or Fix^{-}. (G) At 28 dpi, when Fix^{int} shared a nodule with Fix^{+}, it formed a larger percentage of the bacteroids per nodule than when cohabiting with itself. (H) At 56 dpi, the percentage of bacteroids per mixed nodule which were Fix^{int} was smaller when cohabiting with Fix^{+}, compared to with itself, or Fix^{-}. (A-H) Points represent an average of five technical replicates per strain per independent replicate plant, with each replicate plant consisting of a single nodule. Circles, triangles, and squares indicate data from independent experiments. Black points indicate the overall mean with one standard error, n = 12. (G, H) Dashed line represents where 50% of cells are Fix^{int}. Plants were grown for (A, C, E, H) 28 or (B, D, F, H) 56 days. Values for group means and SEs are in **Table A1.5**.

Together, these results show by 56 dpi, the percentage of bacteria and bacteroids per nodule which were Fix^{int} was smallest when sharing a nodule with Fix^{+}, giving further evidence to support the conditional sanctioning of intermediate strains[108]. However, we did not see similar results in the percentage of CFUs. Additionally, when comparing the absolute numbers of CFUs, bacteria, and bacteroids of Fix^{int} strains in mixed nodules, these values do not differ significantly depending on the cohabiting strain.

4.5 Discussion

In this chapter we demonstrated that sanctioning of indeterminate nodules that are occupied by intermediate or non-fixers results in fewer bacteria and bacteroids per nodule. Additionally, there was some evidence that hosts can sanction intermediate fixers within mixed nodules, though these results were less clear. We tested this by inoculating pea plants with different combinations of otherwise isogenic fixing, intermediate-fixing and non-fixing strains, and used flow cytometry to quantify the number of bacteria and bacteroids in a nodule. To discriminate between the strains *in planta*, and for flow cytometry, we marked and tested strains with different fluorescent proteins. We showed that non-fixers had fewer bacteroids per nodule than fixers, regardless of whether a better strain was available to the plant. However, intermediate-fixing strains had fewer bacteroids only when a better alternative was available to the plant, supporting findings in **Chapter 3** that the plant can conditionally sanction intermediate fixers. We also saw limited evidence for sanctioning within a mixed nodule. Non-fixing strains formed a smaller percentage of the bacteroids in a mixed nodule when sharing with fixing strains. Additionally, when sharing a nodule with a more effective strain, the intermediate strain formed a smaller percentage of the bacteria and bacteroids within the nodule, compared to when sharing with a second intermediate strain or a non-fixer.

First, we determined which fluorescent proteins would be best to use for labelling strains. Fluorescent proteins are needed to identify which strains occupy a nodule, to select the correct nodules for analysis, and to distinguish between multiple strains and background signal in the flow cytometer. We tested a range of fluorescent proteins[145,181,192,193], and determined that GFP and mCherry were the best combination for this study. These could be clearly distinguished in the flow cytometer, and used to quickly identify nodules by

placing root systems on a blue-light transilluminator and imaging with an orange filter[108]. Flow cytometer analyses showed that these fluorescent proteins did not affect the number of bacteria or bacteroids in a strain, and so could be used for competition assays. We also observed that a combination of three colours could be distinguished in the flow cytometer: BFP, YFP and mCherry, which could be used for future experiments comparing more than two strains[189]. It should be noted that other flow cytometers, with other laser and detector combinations, may be better suited to other fluorescent proteins to distinguish cells.

When comparing a mixture of two strains when grown on a slope, each marked with one fluorescent protein, we observed that a large proportion of the rhizobia were showing up as being marked with both fluorescent proteins. This is likely due to two (or more) cells passing through the detector at the same time, which the flow cytometer cannot distinguish, known as doublets[166]. This could be because of the small size of rhizobia[162], which are close to the lower size limit of resolution for the flow cytometer, meaning the detector struggles to distinguish between one cell or multiple cells. However, as this phenomenon is not seen in *Escherichia coli* cells carrying the same fluorescent constructs[189], this difference may instead be due to the production of exopolysaccharides (EPS) by rhizobia, which may cause them to aggregate[102]. The number of double-marked cells was much lower from nodule samples than from the samples grown on a slope, which may be because EPS are expressed highly in culture to enable rhizobia to attach to the root[197].

Doublets can be removed through analysis by comparing the aspect ratios of cells – lower aspect ratios indicate elongated cells, which may be two cells rather than one[166]. However,

as bacteroids are not round, but can form long, branched shapes[55], which may also have a low aspect ratio. Therefore we decided not to filter cells by their aspect ratio in case this excluded larger bacteroids from the analysis. Instead, cells which were double-marked with both fluorescent proteins, and so likely to be doublets, were excluded from flow cytometry analyses for nodule samples.

In addition to testing fluorescent protein colours, we also tested which promoter would be best to label all the cells (both undifferentiated bacteria and bacteroids) within the sample. We tested two synthetic consensus promoters: the strong promoter J23104[191,194], which should be expressed in every rhizobial cell, including both bacteria and bacteroids; and P*nifH*[113], a synthetic *nifH* promoter which is expressed in bacteroids[195] and should therefore label all bacteroid cells. Not all cells from nodule samples were labelled by the P*nifH* promoter, likely due to differential gene expression between bacteria and bacteroids[55]. However, all cells were labelled with the J23104 promoter, so we chose the J23104 promoter for further experiments. Use of this promoter could also allow many further experiments with rhizobia under a wide range of conditions, not just under nitrogen-fixation conditions when *nifH* is expressed.

Using these markers, we then determined the effect of sanctioning on the number of bacteria and bacteroids in single-strain nodules by flow cytometry. At 28 dpi, Fix$^-$ nodules had fewer bacteroids than Fix$^+$ when in co-inoculation, and by 56 dpi, Fix$^-$ nodules had fewer bacteroids than Fix$^+$ when in single or co-inoculation. This shows that Fix$^-$ strains form fewer bacteroids and bacteria than Fix$^+$ in line with histological data[107], possibly because the host plant halts the development of these nodules. However, this does not constitute evidence for conditional sanctioning, as the reduced numbers of Fix$^-$

cells are seen regardless of whether a better strain is available to the host plant or not. In contrast, the number of bacteroids per nodule for Fix^{int} is conditional on the competing strain: by 28 dpi, Fix^{int} forms fewer bacteroids when a better strain is available. This indicates that the number of bacteroids per nodule is not solely determined by the fixation rate of the strain, but is influenced by the host's response to available strains.

For sanctioning within mixed nodules, the evidence for sanctioning of a Fix^- strain was limited. The percentage of bacteroids which were Fix^- was reduced when cohabiting a nodule with Fix^+ at 56 dpi. Additionally, at 56 dpi Fix^- strains formed fewer bacteria and bacteroids than Fix^+ strains when in mixed nodules, but this difference was the same as when a nodule contained only Fix^- or Fix^+ strains. This agrees with work by Regus *et al.* (2017), who showed senescence of Fix^- bacteroids in a mixed nodule[96]. However. we did not observe a reduced number of Fix^- CFUs within a mixed nodule, which conflicts with mixed nodule studies conducted by Daubech *et al.* (2017) who showed changes in the number of CFUs[107]. While the number of Fix^- bacteroids and bacteria was reduced compared to Fix^+, from these results we cannot state that this effect was due to within-nodule conditional sanctioning of Fix^- in response to other available strains, rather than a general response to Fix^- strains. This is because the number of cells did not depend on whether the strain cohabited a nodule with a strain that had the same, or a different, fixation rate.

Interestingly, despite evidence of sanctioning of Fix^- strains in single-strain and mixed-strain nodules, we also observed that in some cases, Fix^- strains had more bacteria than Fix^+ strains. At 28 dpi, we saw that single infection Fix^- nodules had more bacteria than single-infection Fix^+ nodules. Also at 28 dpi, we saw that Fix^- strains had more CFUs

and bacteria per mixed nodule than Fix^+ strains. This is the opposite of what would be expected if the Fix^- strains are sanctioned. However, it is possible we are observing a temporary increase in bacteria numbers for Fix^-, as fewer of these bacteria differentiate into bacteroids, but at a later timepoint, once sanctioning takes hold, these strains have a decreased number of bacteria. This delayed effect of sanctioning on rhizobia numbers is similar to that seen in **3.4.8**.

While there was no evidence of sanctioning of Fix^{int} strains within mixed nodules based on the absolute numbers of CFUs, bacteria, or bacteroids per nodule, we did see evidence of sanctioning within mixed nodules when looking at relative cell numbers. By 56 dpi, the percentage of bacteria and bacteroids per nodule which were Fix^{int} was smaller when sharing a nodule with Fix^+, compared to when sharing a nodule with Fix^- or a differently-marked version of itself. This supports the hypothesis that plants can conditionally sanction poor fixers within mixed nodules.

It is possible that we did not detect an effect of conditional sanctioning on the absolute number of cells due to the large amount of variation in cell numbers between biological replicates. This may be due to differing numbers of cells for each strain entering the mixed nodule when it is formed. Additionally, when selecting nodules, only nodules which had both visible red and green fluorescence were selected. Therefore, a critical number of GFP- or mCherry-marked cells would be needed to observe fluorescence. This could bias the results, as nodules with a more similar number of GFP- and mCherry-marked cells are more likely to be identified as being mixed. Mixed nodules with a too small number of one strain of bacteria may not be identified as being mixed, and so these nodules with

a larger difference in numbers between different rhizobial strains may not have been selected for analysis.

The effect of any bias may be amplified as a result of analysing only one mixed nodule per plant. The experimental design was to analyse five, but at harvest, only one to five mixed nodules per plant could be identified, of which we selected only one. Daubech et *al*. (2017), who observed sanctioning within mixed nodules, analysed three to seven mixed nodules per plant at 14 dpi, and six to eighteen per plant for 35 dpi[107]. Therefore, if the inoculation density was increased to encourage more mixed nodules[105] and then results averaged across multiple mixed nodules per plant, this could reduce the variation between samples and reduce the effect of bias in nodule selection. This could then provide a clearer indication of whether sanctioning can occur within mixed nodules in pea.

Overall, we have demonstrated that sanctioning has an effect on the number of CFUs per nodule, and on the number of bacteria and bacteroids. The impact of sanctioning depends on the strains available to the plant, suggesting that by withholding resources, the host can conditionally regulate the development of nodules and formation of bacteroids. We saw some evidence of sanctioning within mixed nodules, although this was not conclusive. There was no evidence that a poor fixer could prosper via a mixed nodule, as to do so, they would have to gain a fitness advantage when sharing nodules with the fixing strain, and this was not observed in any of our experiments. Further investigation is required to determine whether sanctioning within mixed nodules is common across rhizobial hosts, and if forming mixed nodules could be a way for non-fixing or poorly fixing strains to avoid sanctions and prosper.

Chapter 5 Partner choice and sanctioning in determinate and indeterminate nodules

5.1 Abstract

Plants host nitrogen-fixing rhizobia in specialised root nodules and exchange carbon for nitrogen. However, a non-fixing strain could gain higher fitness by leaving the costly process of nitrogen fixation to others. Plants could discriminate against non-fixing strains by screening them out before they enter nodules (partner choice) or by withholding resources once inside nodules (sanctioning). However, two main nodule types are found in legumes (determinate and indeterminate), and the prevalence of sanctioning versus partner choice may differ between the two. In determinate nodules all rhizobia fix nitrogen and retain the ability to reproduce, whilst in indeterminate nodules, rhizobia terminally differentiate in order to fix nitrogen, so only their undifferentiated clonemates have a life beyond the nodule.

In previous work using pea plants (an indeterminate host) and otherwise isogenic strains of rhizobia, a non-fixing strain was found to be discriminated against via sanctioning rather than partner choice. However, no such clean test has been carried out using a determinate host. Here we compared the response of indeterminate pea (*Pisum sativum*) with determinate bean (*Phaseolus vulgaris*) when inoculated with otherwise isogenic fixing and non-fixing strains of *Rhizobium leguminosarum.* These strains have the same chromosome but different symbiosis plasmids, allowing them to nodulate different host plants.

Neither host showed evidence of partner choice. However, in both pean and bean, non-fixing nodules were smaller than fixing nodules, indicating sanctioning. Additionally, non-fixing nodules from plants co-inoculated with the fixing strain were smaller than non-fixing nodules from plants that were only inoculated with the non-fixer. Furthermore, the fewer nodules the pea plant had, the larger these nodules were, showing that the resources supplied to nodules depends on the strains available and the overall nitrogen status of the plant.

5.2 Overview

The data that support the findings of this chapter are available in the Oxford University Research Archive (DOI: 10.5287/bodleian:zBpJPja9E).

5.3 Introduction

Legume plants host nitrogen-fixing bacteria, known as rhizobia, in specialised root nodules and provide the rhizobia with carbon and other nutrients in return for nitrogen[14]. Rhizobia could be selected to cheat on the plant by leaving the costly process of nitrogen fixation[82] to other strains, and investing more resources in reproduction to gain higher fitness[83,84]. If there is no mechanism to prevent such cheating, this could cause the symbiosis to break down over evolutionary time[85,86]. However, the ability of a non-fixing strain to prosper could differ between the two main nodule types, determinate and indeterminate, due to the different rhizobial life-histories within these nodules[61]. The work in **Chapter 3** and **Chapter 4** focuses on pea, which forms indeterminate nodules, but here we expand this work to include bean, a plant that forms determinate nodules.

Supporting rhizobia is energetically costly for host plants[14,198]. To prevent wasting resources on non-fixing rhizobia, the plant has two potential options: partner choice and sanctioning, although sometimes these terms are used interchangeably[87–89]. We define partner choice as the screening of potential partners for their ability to provide benefits *before* a mutualistic relationship has started[77,90–95], which would result in the host plant forming fewer nodules with less effective strains. In contrast, sanctioning refers to the differential treatment of partners that provide different benefits once the relationship has begun[74,77,89,90,92,93,95–97]. Sanctioning is usually assumed to impose a fitness cost on the recipient, hence we expect sanctioning to result in fewer viable rhizobia per nodule. The mechanism of sanctioning in legumes remains unclear and could be due to the plant withholding resources[108] or through the forced senescence of less effective strains[96].

Different legume species may have evolved different mechanisms to prevent cheats. In particular, rhizobia are known to form two different types of nodule: determinate and indeterminate, depending on their host plant[59,60]. In determinate nodules (like bean), once nodules are formed, rhizobia differentiate into bacteroids which are of a similar size to free-living cells and that retain their ability to reproduce[56,60–62]. In contrast, in indeterminate nodules (like pea), rhizobia are dimorphic: they either do not fix nitrogen and retain their ability to reproduce; or they terminally differentiate into larger non-reproductive nitrogen-fixing bacteroids[56,60–63]. Therefore, while all rhizobia in determinate nodules can both reproduce and fix nitrogen, rhizobia in indeterminate nodules can *either* reproduce *or* fix nitrogen. Here we use bacteroids to refer to differentiated rhizobia in the nodule, bacteria to refer to undifferentiated rhizobia in the nodule, and rhizobia as a general term for all cells.

The outcome of sanctioning could hypothetically differ between the two nodule types, depending on whether sanctioning impacts only bacteroids or whether it also impacts their undifferentiated clonemates. In determinate nodules, sanctions targeted at the bacteroids will also directly reduce rhizobial fitness, as the bacteroids retain their ability to reproduce[125]. Evidence of this was provided by Kiers *et al.* (2003, 2006)[90,106], using *Bradyrhizobium japonicum*, which forms determinate nodules with *Glycine max* (soybean). Nodules were enclosed in a chamber and nitrogen gas replaced with argon, which reduced or prevented nitrogen fixation, and so forced the rhizobia to cheat on the plant. In response, the plant sanctioned the nodules, causing them to be smaller, and when crushed and plated out, these nodules were found to contain fewer viable rhizobia.

In indeterminate nodules, sanctions directed only at bacteroids would prevent the host wasting resources on ineffective strains[74], but it would not affect the fitness of their reproductive clonemates[93,125] which are maintained by a persistent meristem[39,55]. This means that sanctioning in plants with indeterminate nodules may still allow non-fixing strains to prosper, potentially destabilising the mutualism. However, Oono *et al*. (2011)[93] showed a reduction in the number of undifferentiated bacteria for non-fixing nodules in two hosts which form indeterminate nodules (*Pisum sativum* with *Rhizobium leguminosarum*, and *Medicago sativa* with *Sinorhizobium meliloti*). It therefore appears that sanctioning is not just aimed at bacteroids, but can also reduce bacterial fitness.

If legumes are capable of partner choice, then this too could potentially differ between nodule types, due to differences in the signalling mechanisms required for nodule formation[49]. However, it remains unclear how the host plant could assess differences in nitrogen-fixation ability before nodules are formed. Nitrogen-fixation genes are only expressed under the low oxygen concentrations within a nodule[55,57,58], and large-scale mutagenesis and sequencing suggests these genes are only important inside the nodule, not for root colonisation[104]. Despite this, several studies have purported to show that both determinate[89,91,95,99] and indeterminate[77,94,98] host plants preferentially form nodules with more effective strains. However, these studies all used naturally-occurring strains of rhizobia which were collected from the field, which may differ in several traits which affect the ability to form nodules[100], such as growth rate[91], motility[101], root attachment[102], and host-specific signalling genes[49]. It is therefore unclear from these studies whether the observed partner choice was a result of differences in nitrogen-fixation between strains, or differences in other characteristics.

A definitive test of partner choice and sanctioning requires the use of fixing and non-fixing strains, engineered to be isogenic, except for a mutation in a nitrogen-fixation gene, allowing any differences in host response to be attributed to the nitrogen-fixation rate. Such a test was conducted by Westhoek *et al*. (2017)[105], using a fixing strain and an otherwise isogenic non-fixing *nifH* mutant strain, which form indeterminate nodules on pea (*P. sativum*). Here, the ratio of fixing to non-fixing nodules formed by the plant was equal to the ratio of fixing to non-fixing rhizobia used to inoculate the plant, showing no evidence for partner choice. However, nodules containing the non-fixing strain were significantly smaller than those containing the fixing strain, which shows that the plant can sanction ineffective strains. Similar results have been observed for *Cupriavidus taiwanensis*, which forms indeterminate nodules on *Mimosa pudica*[107]. Whilst some evidence of sanctioning[96] and partner choice[127] of a near-isogenic non-fixing mutant has been observed in determinate nodules, a definitive test of partner choice and sanctioning in determinate nodules remains to be carried out.

Here, we compared the evidence for sanctioning and partner choice in determinate and indeterminate nodules using dwarf bean (*Phaseolus vulgaris,* determinate) and pea (*Pisum sativum,* indeterminate). Both plants were inoculated with different combinations of otherwise isogenic fixing (Fix^+) and non-fixing (Fix^-) derivatives of the rhizobial strains *R. leguminosarum* bv. *phaseoli* 4292 (Rlp4292) (bean) and *R. leguminosarum* bv. *viciae* A34 (RlvA34) (pea).

The rhizobial strains RlvA34 and Rlp4292 were selected as they have the same chromosome but differ in the symbiosis (Sym) plasmids that they carry. This plasmid is crucial as it determines which host plant they can nodulate and the type of nodule formed.

Both strains are derived from *R. leguminosarum* bv. phaseoli 8002 (Rlp8002). Rlp4292 is a Rf^r derivative of Rlp8002 containing the Sym plasmid pRP2, allowing it to form determinate nodules on bean[134]. RlvA34 is a St^r derivative of Rlp8002, where the pRP2 plasmid has been cured and replaced with pRL1, allowing it to form indeterminate nodules on pea[136]. RlvA34 and Rlp4292 have previously been used to compare rhizobial transcriptomes[199], symbiotic efficency[163], membrane proteins[124], and the role of carbon storage compounds[200] between pea and bean. The similarity of these two strains allows comparisons to be drawn between nodule types without being confounded by the use of different strains for each host.

To allow testing for partner choice and sanctioning, Fix^- versions of RlvA34 and Rlp4292 were generated by the insertion of a Ω spectinomycin resistance (Sp^r) cassette in the *nifK* gene, a key gene for nitrogen fixation[57], a singular copy of which is found in both RlvA34 and Rlp4292. *nifK* was chosen rather than *nifH* as in Westhoek *et al.* (2017)[105], **Chapter 3**[108] and **Chapter 4**, as unlike Rlv3841, Rlp4292 contains three copies of *nifH* (two complete genes and one pseudogene)[199]. This gives a set of near-isogenic Fix^+ and Fix^- rhizobia which can be used to compare partner choice and sanctioning in determinate and indeterminate nodules.

To test for partner choice and sanctioning, plants were simultaneously co-inoculated with two different strains. In order to identify the occupant of each nodule, strains were labelled with either *gusA* or *celB* markers. These markers are chromogenic, and post-harvest GusA and CelB chromogenic staining[135] was used to distinguish between strains, allowing the counting of nodules and measuring of nodule size. We tested these markers

to ensure they had no deleterious effect on the rhizobia which could affect their ability to compete for nodules or their rate of growth and reproduction within nodules.

We then tested whether host plants with determinate and indeterminate nodules discriminate between rhizobial strains using partner choice or sanctioning. Specifically, we tested whether host plants form proportionally more nodules with Fix^+ strains compared to Fix^- strains (partner choice) or whether nodules containing Fix^+ strains are larger than those containing Fix^- strains (sanctioning). We tested for partner choice by inoculating host plants with different ratios of Fix^+:Fix^- strains, and determining whether either strain formed more nodules that expected. We tested for sanctioning by comparing the sizes of Fix^+ and Fix^- nodules, as sanctioned strains are often observed to have smaller nodules[88,90,93,105,106,108]. We determined whether Fix^- nodules were smaller than Fix^+ nodules when co-inoculated on the same plant. We also tested whether Fix^- nodules are smaller when competing against Fix^+, than when competing against itself. We carried out the same experiments with both pea and bean to see whether the mechanisms of partner choice and sanctioning differ between nodule types, which might have implications for the long-term stability of the symbiosis.

5.4 Results

5.4.1 Generation of non-fixing strains for competition assays

5.4.1.1 Creation of *nifK*::ΩSp^r pJQ200SK plasmids for RlvA34 and Rlp4292

We created Fix$^-$ mutants of RlvA34 and Rlp4292 by deleting *nifK*, a key gene for nitrogen fixation[57]. *nifK* was chosen over *nifH*, as seen in **Chapter 3** and **Chapter 4**, as both RlvA34 and Rlp4292 contain only one copy of *nifK*[199]. In-frame mutagenesis of the *nifK* gene in RlvA34 and Rlp4292 was carried out using the plasmid pJQ200SK. Here the flanking regions of the *nifK* gene surrounding an ΩSp^r cassette (*nifK*::ΩSp^r) were inserted into the *Rhizobium* strains via a pJQ200SK plasmid. The flanking regions would allow homologous recombination of the *nifK*::ΩSp^r with the host *nifK* flanking regions, where a double-crossover event would replace *nifK* with *nifK*::ΩSp^r. To mutate both strains, two *nifK*::ΩSp^r pJQ200SK plasmids were generated, containing the flanking regions for either RlvA34 or Rlp4292 *nifK*.

To generate the *nifK*::ΩSp^r plasmids, genomic DNA was extracted from RlvA34 and Rlp4292, and the *nifK* gene plus 1 kb of flanking region on either side was amplified by PCR, using primers oxp1857 and oxp1858 for RlvA34, and oxp1859 and oxp1860 for Rlp4292 (**Figure 5.1*A***). The forward primers had an 18 bp extension containing an I-*Sce*I homing endonuclease recognition site. The resultant PCR product was cloned into the blunt vector pJET1.2 (**Figure 5.1*B***), transformed into *E. coli* DH5α competent cells, and selected for on LB agar with carbenicillin. Eight individual colonies per plasmid were checked by colony PCR using pJET1.2 forward and reverse primers to amplify the *nifK* and flanking regions cloned into pJET1.2. Colonies with the expected band size (3653 bp for RlvA34, 3673 bp for Rlp4292) had their plasmids extracted and digested with

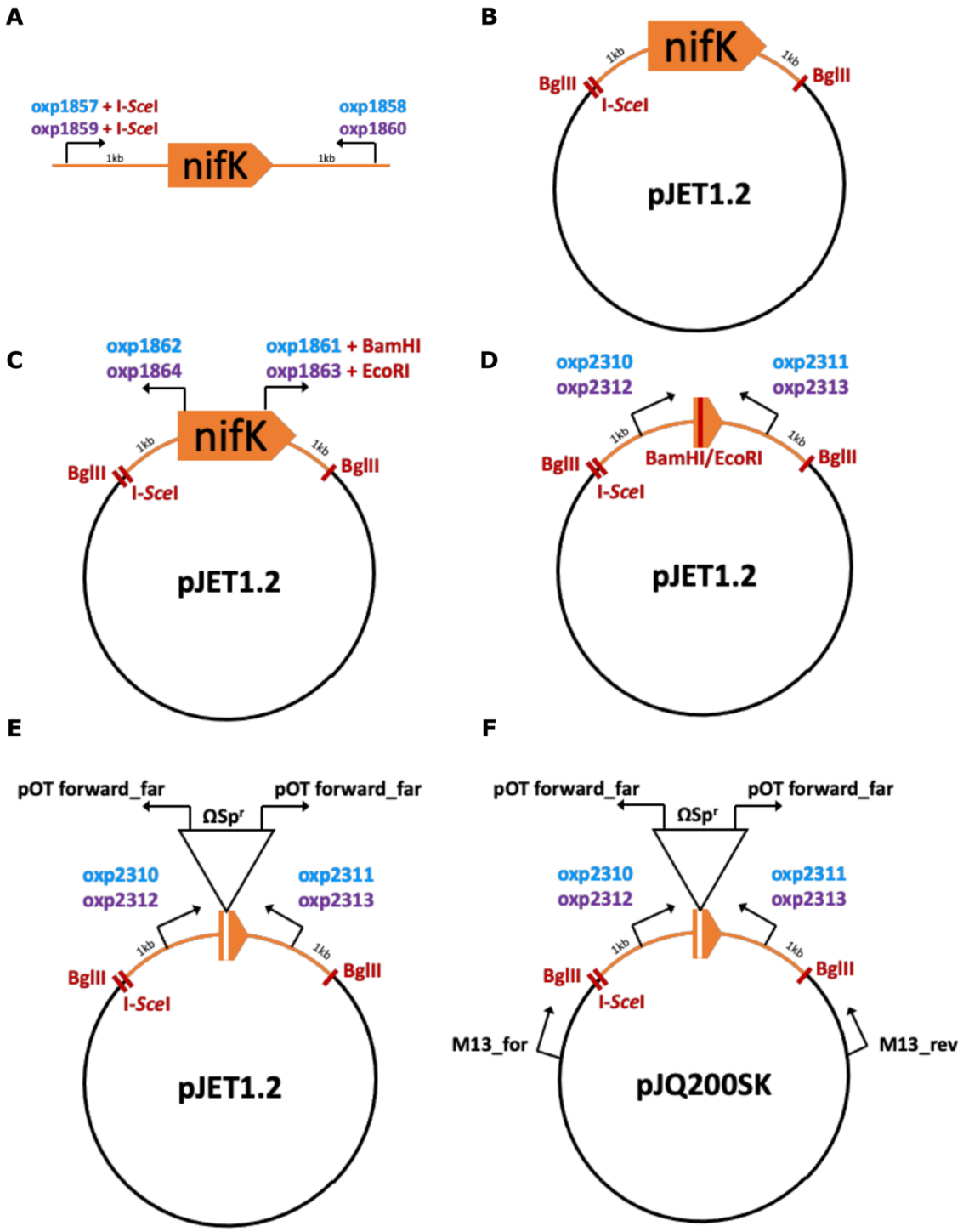


Figure 5.1 – Generation of *nifK*::ΩSp^r pJQ200SK plasmids for RlvA34 and Rlp4292. (A) The *nifK* gene plus 1 kb of flanking region on either side was amplified by PCR. (B) The resultant PCR product was cloned into the blunt vector pJET1.2. (C) Inverse PCR was used to remove most of the *nifK* gene, but leave ~ 20 bp at each edge, and the flanking regions, in pJET1.2. (D) The inverse PCR product was re-ligated using BamHI or EcoRI. (E) ΩSp^r was cloned into the plasmid by digestion and re-ligation. (F) The *nifK*::ΩSp^r constructions were added to the pJQ200SK suicide plasmid by digestion and re-ligation. Blue indicates primers used for the RlvA34 plasmid, purple indicates primers used for the Rlp4292 plasmid, and red indicates restriction enzyme recognition sites.

restriction enzymes to confirm the correct sizes (RlvA34 with EcoRI and Rlp4292 with PstI). Plasmids with the expected bands (4051 bp, 1917 bp and 465 bp for RlvA34 as 110 bp was too small to see; and 3420 bp, 2217 bp and 893 bp for Rlp4292) were sent for sequencing with pJET1.2 forward and reverse primers to confirm the correct assembly.

Inverse PCR was then carried out on both *nifK* regions in pJET1.2, to remove most of the *nifK* gene, but leave ~ 20 bp at each edge, and the flanking regions, in pJET1.2 (**Figure 5.1*C***). PCR was used to amplify the edges of *nifK*, the flanking regions, and the pJET backbone, using primers oxp1861 and oxp1862 with an added BamHI site for RlvA34, and oxp1863 and oxp1864 with an added EcoRI site for Rlp4292. The inverse PCR product was re-ligated using BamHI or EcoRI, transformed into *E. coli* DH5α competent cells, and selected for on LB agar with carbenicillin (**Figure 5.1*D***). Eight individual colonies per plasmid were checked by colony PCR using pJET1.2 forward and reverse primers to amplify the edges of *nifK* and flanking regions cloned into pJET1.2. Colonies with the expected band size (2201 bp for RlvA34, 2186 bp for Rlp4292) had their plasmids extracted and digested with restriction enzymes to confirm the correct sizes (RlvA34 with BamHI and Rlp4292 with EcoRI). Plasmids with expected bands (5061 bp for RlvA34, 5046 bp for Rlp4292) were sent for sequencing with mapping primers to confirm that the deletions were in-frame (oxp2310 and oxp2311 for RlvA34, oxp2312 and oxp2313 for Rlp4292).

ΩSp^r was then cloned into the plasmid (**Figure 5.1*E***). Each plasmid was digested between the two flanking regions (BamHI for RlvA34, EcoRI for Rlp4292), the band size confirmed (5061 bp for RlvA34, 5046 bp for Rlp4292) and the digestion product purified. The pHP45Ω-Sp plasmid[139], which contains the ΩSp^r cassette, was also digested with

BamHI or EcoRI for ligating into each plasmid, and the band size confirmed (the ΩSp^r cassette and the plasmid backbone, 2060bp and 2351bp for BamHI, 2080bp and 2331bp for EcoRI). The digestion was purified, then digested again with PstI to digest the plasmid backbone leaving 766 bp, 1589 bp and 2060 bp fragments for BamHI; and 756 bp, 1579 bp and 2080 bp for EcoRI. The ~2kb fragment corresponding to the ΩSp^r cassette was extracted from a gel and purified. The ΩSp^r cassette and the corresponding digested pJET1.2 plasmids were then ligated, transformed into *E.coli* DH5α competent cells, and selected for on LB agar with carbenicillin and spectinomycin. The Rlp4292 *nifK*::ΩSp^r construction was screened by colony PCR as before, using primers oxp1863 and pOT forward_far (5179 bp). The RlvA34 *nifK*::ΩSp^r construction was screened by restriction enzyme digest, using BglII to confirm band sizes of 2932 bp and 4189 bp.

The *nifK::*ΩSp^r constructions were then added to the pJQ200SK suicide plasmid[142] (**Figure 5.1*F***). The pJQ200SK plasmid was digested with BamHI, leaving a BglII overhang, and PCR purified. The digested pJQ200SK was then dephosphorylated to reduce self-ligation. The plasmids containing *nifK::*ΩSp^r for RlvA34 and Rlp4292 were digested with BglII, and the band sizes confirmed (2932 bp and 4189 bp for RlvA34, 2932 bp and 4194 bp for Rlp4292). The ~4 kb bands, corresponding to the ΩSp^r cassettes with *nifK* flanking regions, were extracted and purified. The two fragments were ligated with a molar ratio of 3:1 of insert to plasmid, transformed into *E. coli* DH5α competent cells, and selected for on LB agar with gentamycin, spectinomycin and X-gal for blue-white selection. White colonies were checked by colony PCR using primers M13_for with pOT forward_far, and M13_rev with pOT forward_far. The M13 primers bind in the vector backbone with pOT forward_far in the ΩSp^r cassette. Colonies with the expected band sizes (1286 bp and 1357 bp for RlvA34, 1321 bp and 1327 bp for Rlp4292) had

their plasmids extracted and digested with EcoRI to confirm (3221 bp, 2964 bp, 2710 bp, and 570 bp for RlvA34 as 110 bp too faint to see; 3714 bp, 2710 bp, 2018 bp, and 1072bp for Rlp4292). Successful digestions were then sequenced with M13_uni F and M13_rev to confirm the correct sequences. Correct *nifK*::ΩSp^r in pJQ200SK plasmids were stocked as pOPS0997 for Rlp4292 (**Figure 5.2*A***) and pOPS0998 for RlvA34 (**Figure 5.2*B***).

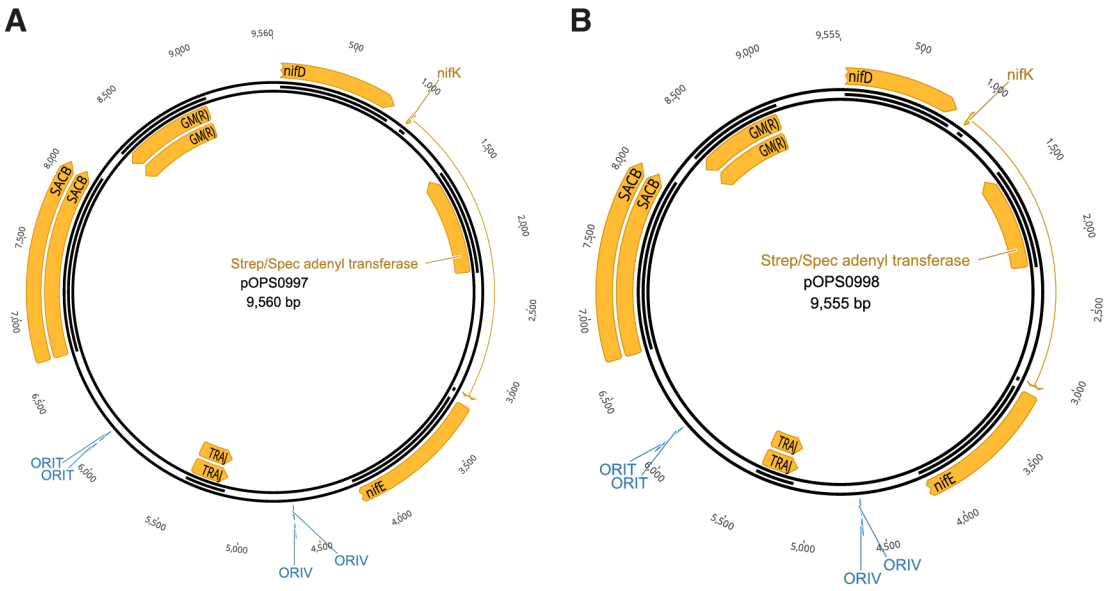


Figure 5.2 – Plasmids containing *nifK*::ΩSp^r. (A) Plasmid pOPS0997 for Rlp4292 mutagenesis. (B) Plasmid pOPS0998 for RlvA34 mutagenesis.

5.4.1.2 Generation of *nifK*::ΩSp^r RlvA34 and Rlp4292 using pJQ200SK mutagenesis

We then isolated Fix$^-$ derivatives of RlvA34 and Rlp4292 using the *nifK*::ΩSp^r pJQ200SK plasmids pOPS0997 and pOPS0998. The *nifK*::ΩSp^r had homology to the host *nifK* flanking regions, where a double-crossover event replaced *nifK* with *nifK*::ΩSp^r. The remaining pJQ200SK plasmid was then chased out.

pOPS0997 and pOPS0998 were first transformed into *E. coli* ST18 competent cells, to aid with the conjugation into RlvA34 and Rlp4292 and subsequent selection, and selected

for on LB agar with gentamicin, spectinomycin and 5-aminolevulinic acid (ALA, required for ST18 growth). Colonies were checked by colony PCR using primers M13_for with pOT forward_far, and M13_rev with pOT forward_far, again to amplify each flanking region, as the M13 primers bind in the vector backbone with pOT forward_far in the ΩSp^r cassette. The expected band sizes were 1286 bp and 1357 bp for RlvA34, 1321 bp and 1327 bp for Rlp4292.

pOPS0997 and pOPS0998 in ST18 cells were then conjugated into RlvA34 and Rlp4292 by triparental mating, with TY agar supplemented with ALA for the mating plate. Conjugations were selected for on TY agar with streptomycin for RlvA34, rifampicin for Rlp4292, and added spectinomycin to select for *nifK*::ΩSp^r. Colonies were screened by colony PCR to confirm double crossover events with the host DNA, using mapping primers which bind between 200-500 bp outside of the flanking regions and can therefore test for double crossover events, in combination with pOT forward_far which binds at both ends of the ΩSp^r. These primers were oxp2465 with pOT forward_far and oxp2466 with pOT forward_far for RlvA34, and oxp2463 with pOT forward_far and oxp2464 with pOT forward_far for Rlp4292. Bands of 1321 bp and 1264 bp were expected for RlvA34, and 1268 bp and 1306 bp with Rlp4292.

The pDAI-*Sce*I plasmid[138] was then conjugated into the putative *nifK* deletions to target the *Sce*I homing endonuclease site in the pJQ200SK plasmids. This would be lethal to any cells with a remaining pJQ200SK plasmid, therefore selecting for cells where the pJQ200SK plasmid had been cured. The pDAI-*Sce*I plasmid was conjugated in via triparental mating, and successful colonies were patch plated onto TY agar, and TY agar supplemented with gentamycin. Colonies which were not resistant to gentamycin were selected, as these would have lost their pJQ200SK plasmid. Colonies were screened by

colony PCR to confirm if the resultant strains were a *nifK*::ΩSp^r or a wild-type revertant. Primers oxp2465 with pOT forward_far and oxp2466 with pOT forward_far were used for RlvA34, and oxp2463 with pOT forward_far and oxp2464 with pOT forward_far for Rlp4292, with expected band sizes as before.

The loss of the pDAI-*Sce*I plasmid was then selected for. *nifK*::ΩSp^r colonies were patch plated onto TY agar and TY agar with tetracycline, as tetracycline sensitivity would indicate the loss of the pDAI-*Sce*I plasmid. Colonies were again screened by colony PCR to confirm they were *nifK*::ΩSp^r, using primers oxp2465 with pOT forward_far and oxp2466 with pOT forward_far for RlvA34, and oxp2463 with pOT forward_far and oxp2464 with pOT forward_far for Rlp4292, with expected band sizes as before.



Figure 5.3 – Double-crossover events to replace *nifK* with *nifK*::ΩSp^r. (A) Plasmid pOPS0998 was used to insert a ΩSp^r cassette in RlvA34 to make *nifK*::ΩSp^r strain OPS1480. (B) Plasmid pOPS0997 was used to insert a ΩSp^r cassette in Rlp4292 to make *nifK*::ΩSp^r strain OPS1457. (A, B) Red indicates the areas for crossover events.

Putative *nifK*::ΩSp^r colonies were mapped by sequencing using primers oxp2465 with pOT forward_far and oxp2466 with pOT forward_far for RlvA34, and oxp2463 with pOT forward_far and oxp2464 with pOT forward_far for Rlp4292. The primer oxp2465

produced poor quality reads of the cross-over region as it was located too close, so a new mapping primer oxp2637 was used. Successful *nifK*::ΩSp^r strains were stocked as OPS1480 for RlvA34 (**Figure 5.3*A***) and OPS1457 for Rlp4292 (**Figure 5.3*B***).

5.4.2 Assessment of non-fixing strains

To confirm that the *nifK*::ΩSp^r insertion rendered OPS1480 and OPS1457 non-fixing, we measured the rates of acetylene reduction of both strains using single inoculations.

Pea plants were inoculated with either Fix$^+$ (RlvA34), or Fix$^-$ (OPS1480). One pre-germinated pea seed was potted per 500 ml pot containing a 1:1 mixture of silver sand and fine vermiculite and inoculated with 1 x 10^8 cells per pot. Bean plants were inoculated with either Fix$^+$ (Rlp4292), or Fix$^-$ (OPS1457). Three pre-germinated bean seeds were potted per 1 L pot containing a 1:1 mixture of silver sand and fine vermiculite and inoculated with 1 x 10^8 cells per pot. In two independent experiments, four replicate plants per treatment and four water controls of each host plant were grown in a fully randomised design, 28 days for pea and 35 days for bean. After seven days plants were thinned to one per pot.

Acetylene reduction rates were measured, the number of nodules counted, and the shoot dry biomasses measured. Data were analysed separately for pea and bean. Nitrogen fixation rates and shoot dry biomass were analysed as a function of rhizobial strain using a linear model. Nodule number was analysed as a function of rhizobial strain using a generalised linear model with a quasipoisson error distribution.

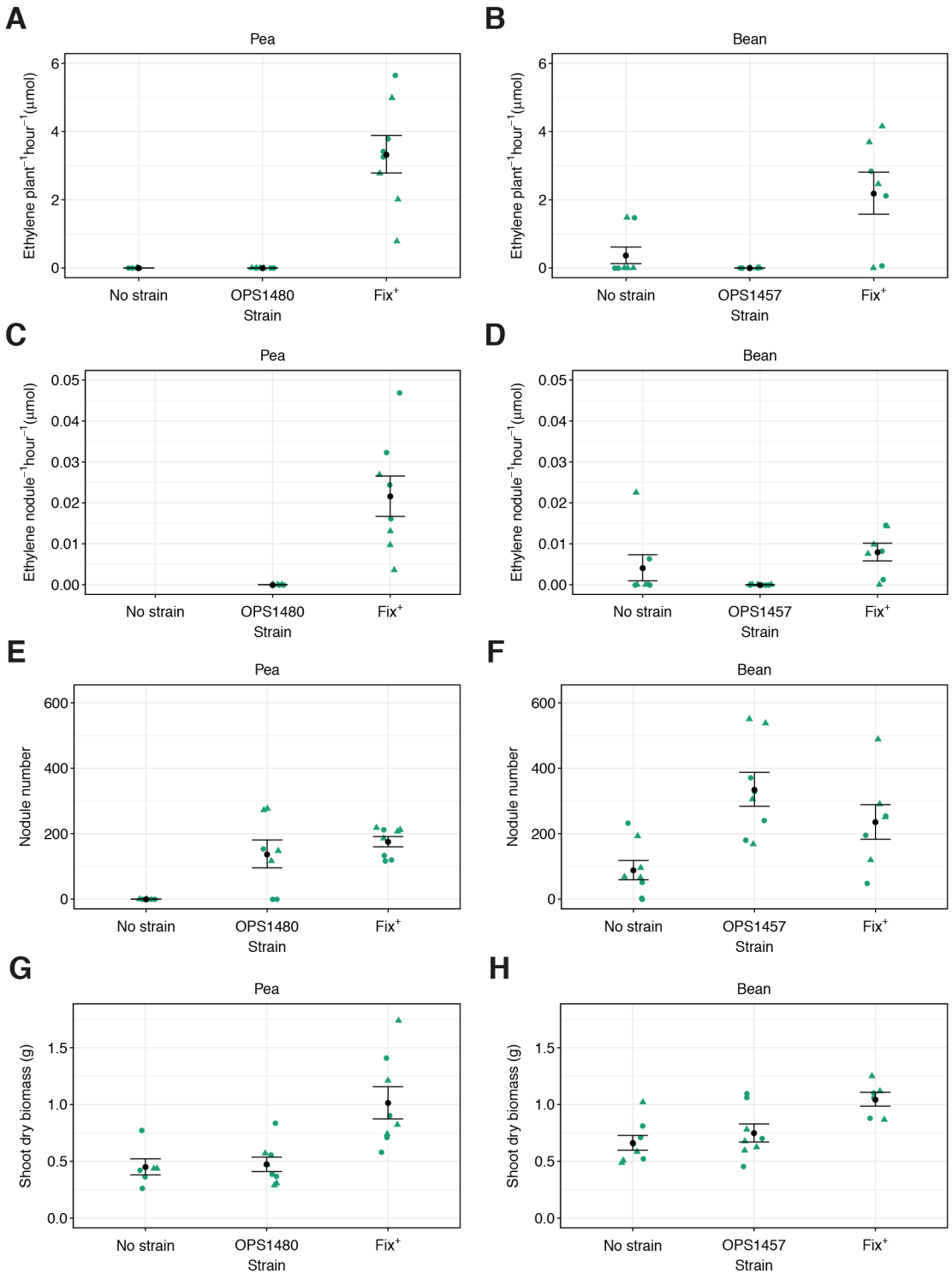


Figure 5.4 – The nitrogen-fixing ability of Fix^{+} strains and their derived mutants. (A) OPS1480 and (B) OPS1457 do not fix nitrogen. (C) OPS1480 and (D) OPS1457 do not fix nitrogen when expressed at a per nodule rate. (E) OPS1480 and (F) OPS1457 both form as many nodules as Fix^{+} on their appropriate hosts. (G) OPS1475 and (H) OPS1457 both have a lower shoot biomass than Fix^{+} on their appropriate hosts, but this is similar to the water controls. (A-D) Points represent an average of three technical replicates per independent replicate plant, (E-H) points represent each independent replicate plant. (A-H) Circles and triangles indicate data from independent experiments. Black points indicate the overall mean with one standard error. (A, C, E, G) Pea plants were grown for 28 days, n = 6-8. (B, D, F, H) Bean plants were grown for 35 days, n = 7-8.

Acetylene reductions revealed that neither OPS1480 nor OPS1457 fixed nitrogen. Per plant, OPS1480 fixed a mean of 0.0000 μmol ethylene plant^{-1} hour^{-1} (95% CI [-0.6979, 0.6979], **Figure 5.4*A***), and OPS1457 fixed a mean of 0.0000 μmol ethylene plant^{-1} hour^{-1} (95% CI [-0.7227, 0.7227], **Figure 5.4*B***). When expressed per nodule, OPS1480 fixed a mean of 0.0000 μmol ethylene nodule^{-1} hour^{-1} (95% CI [-0.0095, 0.0095], **Figure 5.4*C***), and OPS1457 fixed a mean of 0.0000 μmol ethylene nodule^{-1} hour^{-1} (95% CI [-0.0042, 0.0042], **Figure 5.4*D***).

Pea plants inoculated with OPS1480 formed a mean of 199 nodules, a similar number to plants inoculated with Fix^{+} (mean 176, ns difference of 13 to OPS1480, risk ratio [RR] 0.88, 95% CI [0.47, 1.67], **Figure 5.4*E***). Bean plants inoculated with OPS1457 formed a mean of 336 nodules, a similar number to plants inoculated with Fix^{+} (mean 236, ns difference of 100 to OPS1457, RR 0.70, 95% CI [0.41, 1.19], **Figure 5.4*F***).

Pea plants inoculated with OPS1480 had a mean shoot dry biomass of 0.47 g, similar to water controls (mean 0.45 g, ns difference of 0.02, 95% CI [-0.29, 0.34], **Figure 5.4*G***), and substantially less than Fix^{+} plants (mean 1.02 g, 0.54 less, 95% CI [0.25, 0.84], **Figure 5.4*G***). Bean plants inoculated with OPS1457 had a mean shoot dry biomass of 0.75 g, similar to water controls (mean 0.66 g, ns difference of 0.09, 95% CI [-0.17, 0.34], **Figure 5.4*H***), and substantially less than Fix^{+} plants (mean 1.12 g, 0.41 less, 95% CI [0.14, 0.68], **Figure 5.4*H***).

As neither OPS1480 or OPS1457 fixed nitrogen, this demonstrates that the *nifK*::ΩSp^{r} insertions in RlvA34 and Rlp4292 successfully resulted in non-fixing strains (Fix^{-}).

5.4.3 Generation of marked strains for competition assays

5.4.3.1 Creation of mini-Tn*7* *gusA* and *celB* markers

To assess sanctioning and partner choice in determinate and indeterminate nodules, we needed to be able to distinguish between Fix^+ and Fix^- nodules in order to count the number of nodules and measure nodule sizes. To enable this, we created and inserted *gusA* and *celB* markers into Fix^+ and Fix^- derivatives of RlvA34 and Rlp4292. This allowed nodules containing different strains to be distinguished by GusA and CelB sequential staining, rendering nodules either magenta or blue[135,158].

Plasmid-based pOGG026 *gusA* and *celB* markers were initially tested. This broad host-range marker system has been previously used in a variety of species[108,113,144], including RlvA34 and Rlp4292[113]. However, here, experiments using RlvA34 and Rlp4292 marked with this plasmid often resulted in chlorotic and small non-fixing nodules, with host plants which were yellow and smaller than expected, similar to when inoculated with a non-fixing strain. We suspect this is due to an incompatibility between the host strain and either the RK2 replicon or neomycin resistance cassette in pOGG026, though this was not further investigated. As an alternative to plasmid-based pOGG026 marking, mini-Tn*7* marking was attempted, as this inserts the marker genes into the chromosome[149,151] and should therefore avoid any issues with plasmid incompatibility.

The marker plasmids were assembled using Golden Gate cloning[130]. This involves using genetic modules, which when digested by type IIs restriction enzymes, have sticky-ends which result in the DNA fragments assembling in the correct order in a vector[130,131]: the PU module (promoter with the ribosome binding site), SC module (ORF), and the T module (terminator). The PU module pOGG043 was used, containing a synthetic

consensus *nifH* promoter (Ps*nifH*) which is expressed in nodules in a variety of *Rhizobium* species including RlvA34 and Rlp4292[113]. The SC modules were either pOGG083[144] for the *gusA* plasmid, or pOGG050[144] for the *celB* plasmid. The T module pOGG157 contained the DT16 short terminator[146]. The mobilisable vector pOGG276 was used, which can host sequences for mini-Tn*7* insertion. This vector contains Amp^R in the vector backbone to select for the vector, and Gm^R in the genomic insert to select for insertion in the chromosome[147].

Golden gate reactions were carried out for both plasmids[131]. The reaction mix was transformed into EC100D™ pir-116 competent cells, and selected for on LB agar with gentamycin and X-gal. White colonies were selected, which indicates the displacement of *lacZα* in the cloning site of the vector, which would otherwise result in blue colonies[130].

Eight individual colonies per conjugation were checked by colony PCR. Two colonies of the empty vector pOGG276 were used as negative controls. Primers oxp3062 and oxp3063 were used to amplify the assembly region, which was 804 bp in the vector, 2317 bp in the *gusA* plasmid and 1924 bp in the *celB* plasmid. All tested colonies showed the expected band size. Plasmids were amplified by PCR using primers oxp3062 and oxp3063, the PCR product purified, and sent for sequencing with primers oxp3062 and oxp3063. One plasmid of each type showed the correct sequence across the assembly junctions. These plasmids were stocked, with the *gusA* plasmid as pOPS1548 (**Figure 5.5*A***), and the *celB* plasmid as pOPS1549 (**Figure 5.5*B***).

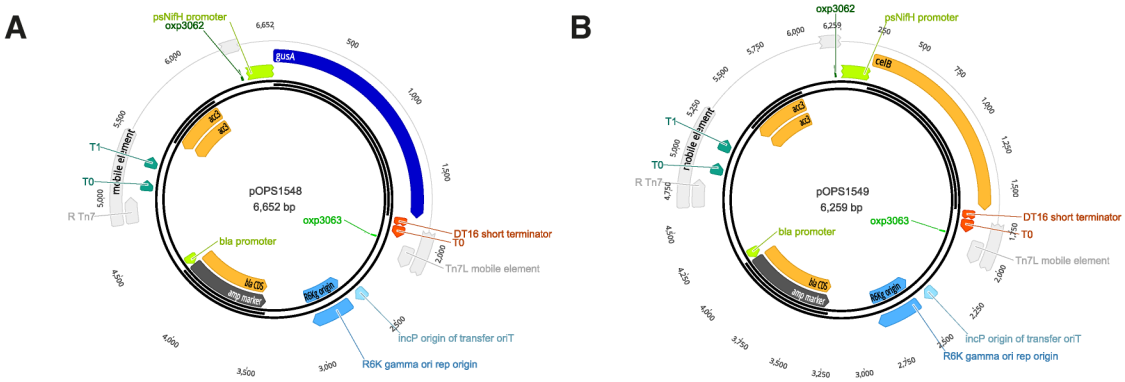


Figure 5.5 – *gusA* and *celB* marker plasmids. (A) Plasmid pOPS1548 contains *gusA* under Ps*nifH* promotion. (B) Plasmid pOPS1549 contains *celB* under Ps*nifH* promotion.

5.4.3.2 Marking strains with *gusA* and *celB*

We then added the *gusA* and *celB* makers to the RlvA34 and Rlp4292 strains by mini-Tn*7* insertion into the chromosome[149,151]. The *gusA* and *celB* donor plamids (pOPS1548 and pOP1549) were transformed into ST18 competent cells, before being conjugated into both Fix$^+$ strains (RlvA34 and Rlp4292) and both Fix$^-$ strains (OPS1480 and OPS1457) using pTNS3[149] and selected for on TY agar with nitrofurantoin and gentamycin.

Four individual colonies per conjugation were checked by colony PCR. Two colonies of each recipient strain and each donor plasmid were used as negative controls. One reaction used primers oxp2327 and oxp3060 to amplify a ~250 bp region from the left Tn*7* end into the chromosome. The second reaction used primers oxp2328 and oxp3061 to amplify a ~500 bp region from the right Tn*7* end into the chromosome. A correct colony for each strain was identified and stocked with RlvA34 Fix$^+$ *gusA* as OPS2306, RlvA34 Fix$^+$ *celB* as OPS2307, RlvA34 Fix$^-$ *gusA* as OPS2308, RlvA34 Fix$^-$ *celB* as OPS2309, Rlp4292 Fix$^+$ *gusA* as OPS2373, Rlp4292 Fix$^+$ *celB* as OPS2348, Rlp4292 Fix$^-$ *gusA* as OPS2374, and Rlp4292 Fix$^-$ *celB* as OPS2349.

5.4.3.3 In planta testing of *gusA*- and *celB*-marked strains

We tested the *gusA*- and *celB*-marked strains *in planta* to determine whether they could nodulate plants, and whether, after harvesting, GusA and CelB sequential staining could be used to distinguish between nodules containing different strains.

Pea plants were inoculated with single inoculations of unmarked Fix^+ (RlvA34), or Fix^- (OPS1480); *gusA*-marked Fix^+ (OPS2306), or Fix^- (OPS2308); or *celB*-marked Fix^+ (OPS2307), or Fix^- (OPS2309). One pre-germinated pea seed was potted per 500 ml pot containing medium vermiculite and inoculated with ~1 x 10^8 cells per seed. Bean plants were inoculated with single inoculations of unmarked Fix^+ (Rlp4292), or Fix^- (OPS1457); *gusA*-marked Fix^+ (OPS2373), or Fix^- (OPS2374); or *celB*-marked Fix^+ (OPS2348), or Fix^- (OPS2349). Two pre-germinated bean seeds were potted per 1 L pot containing fine vermiculite and inoculated with ~1 x 10^8 cells per seed. Two replicate plants per treatment and two water controls per host plant were grown in a fully randomised design, 28 days for pea and 35 days for bean. After seven days plants were thinned to one per pot. At harvest, roots were sequentially stained for GusA and CelB[135,158], and photographed.

Images of the pea and bean rooting systems after undergoing GusA and CelB staining are shown in **Figure 5.6**. Nodules of *gusA*-marked strains were magenta (**Figure 5.6*B, E, I, L*)** whilst nodules of *celB*-marked strains were blue (**Figure 5.6*C, F, J, M***). Nodules of unmarked strains were a beige or brown colour (**Figure 5.6*A, D, H, K***), and the uninoculated controls did not have any nodules (**Figure 5.6*G, N***). This demonstrates that GusA and CelB staining can be used to distinguish between strains when labelled with either *gusA* or *celB*.

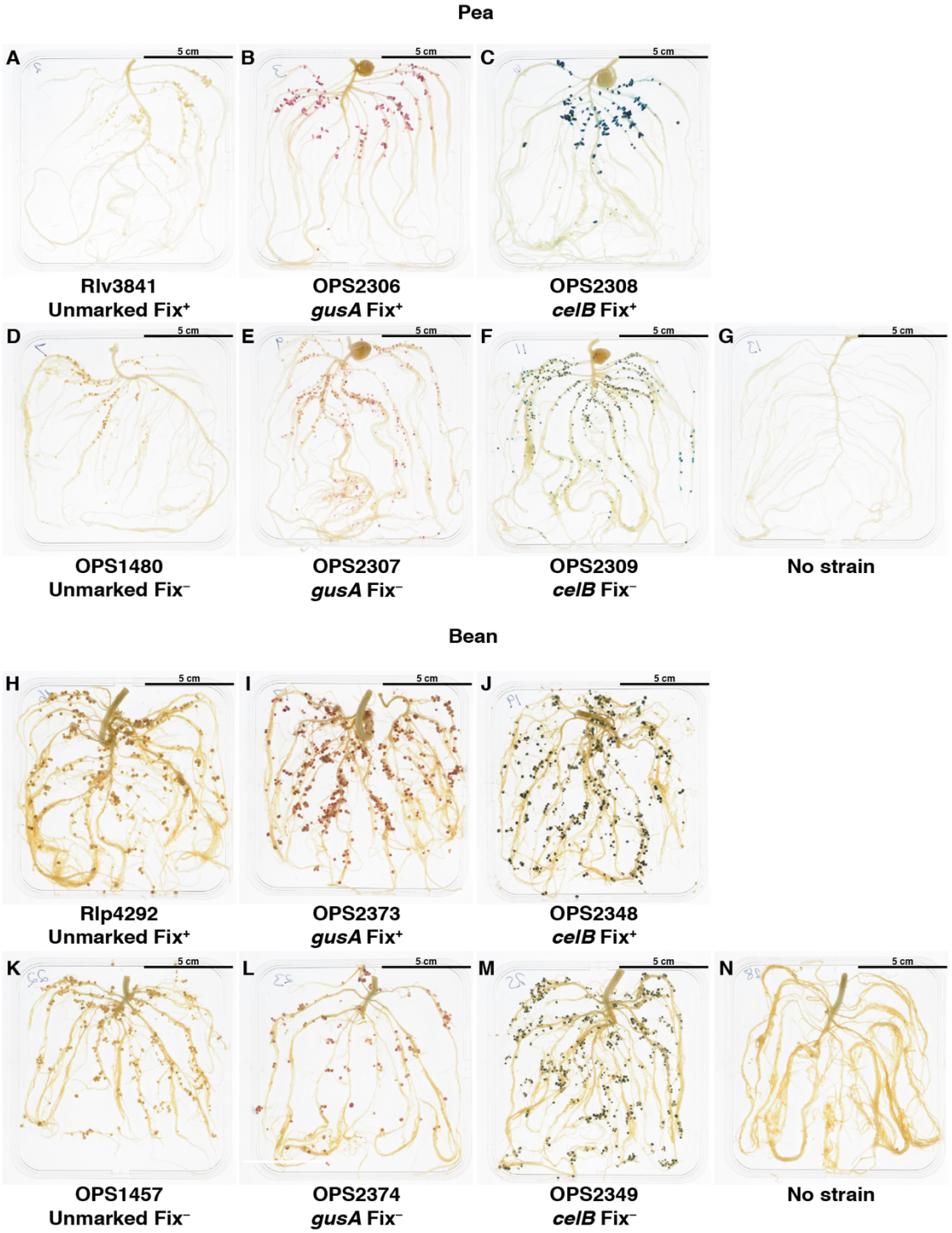


Figure 5.6 – Root systems inoculated with unmarked, *gusA*-marked or *celB*-marked Fix$^+$ and Fix$^-$. (A-N) Root systems underwent sequential GusA and CelB staining. Scale bar indicates 5 cm. Images shown are representative of two biological replicates. (A-G) Pea plants were harvested at 28 dpi. (H-N) Bean plants were harvested at 35 dpi.

5.4.3.4 Growth rate of *gusA*- and *celB*-marked strains

As the *gusA*- and *celB*-marked strains were to be used in competition assays, we tested whether the marker genes impacted the growth rate of strains, as this might affect the outcomes of competition assays. We measured the generation times of all the marked strains, and their unmarked parental strains, using standard growth curves. One replicate of each strain was measured in three independent experimental repeats. Generation times were analysed as a function of strain using separate linear models for RlvA34- and Rlp4292-based strains, excluding water controls as these showed no growth.

The RlvA34-based strains had a mean generation time of 3.33 hours, which did not vary significantly among strains ($F_{5,12}$ = 0.5197, p = 0.7570, **Figure 5.7*A***). Similarly, the Rlp4292-based strains had a mean generation time of 3.13 hours, which did not vary significantly among strains ($F_{5,12}$ = 1.734, p = 0.2014, **Figure 5.7*B***). This demonstrates that the *gusA* and *celB* marker genes did not have a deleterious effect on the growth rate of any of the strains under the *in vitro* conditions tested.

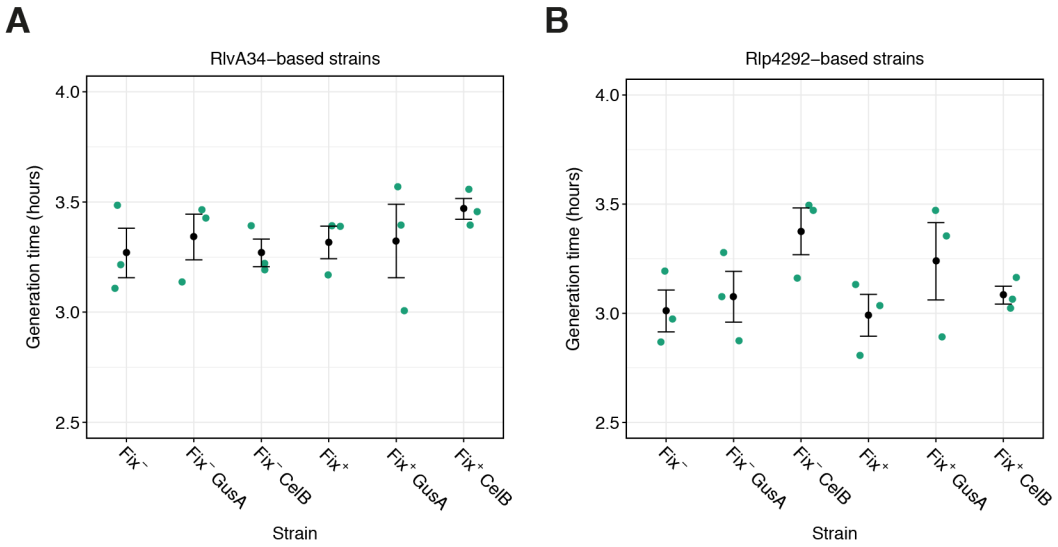


Figure 5.7 – The effect of *gusA* and *celB* marker genes on generation time. Generation time did not vary significantly among unmarked, *gusA*-marked, or *celB*-marked Fix^+, or Fix^- (A) RlvA34-based or (B) Rlp4292-based strains. (A, B) Points represent independent replicates. Black points indicate the overall mean with one standard error, n = 3.

5.4.3.5 Nitrogen fixation of *gusA*- and *celB*-marked strains

As the *gusA* and *celB* marker genes did not impact the growth rate of the RlvA34- or Rlp4292-based strains *in vitro*, we then compared the effects of the marker genes on the nitrogen fixation rates of the strains. It was important to determine that the marker genes did not have a differential effect on the fixation rates of the strains, as any differences seen in competition assays should be due to differences in the parental strains (Fix^+ or Fix^-), not the marker used.

Pea plants were inoculated with single inoculations of unmarked Fix^+ (RlvA34), or Fix^- (OPS1480); *gusA*-marked Fix^+ (OPS2306), or Fix^- (OPS2308); or *celB*-marked Fix^+ (OPS2307), or Fix^- (OPS2309). Two pre-germinated pea seeds were potted per 500 ml pot containing medium vermiculite and inoculated with ~1 x 10^8 cells per seed. Bean plants were inoculated with single inoculations of unmarked Fix^+ (Rlp4292), or Fix^- (OPS1457); *gusA*-marked Fix^+ (OPS2373), or Fix^- (OPS2374); or *celB*-marked Fix^+ (OPS2348), or Fix^- (OPS2349). Three pre-germinated bean seeds were potted per 1 L pot containing fine vermiculite and inoculated with ~1 x 10^8 cells per seed. In two independent experiments, four replicate plants per treatment and four water controls per host plant were grown in a fully randomised design, 28 days for pea and 35 days for bean. After seven days plants were thinned to one per pot.

At harvest, acetylene reduction rates were measured; and nodules were picked, counted, and weighed. Water controls were excluded from the analysis. Data were analysed separately for pea and bean. To test for the effect of marker, nitrogen-fixation rates were analysed as a function of the parent strain (Fix^+ and Fix^-) and the marker gene (*gusA* and *celB*) using a linear model.

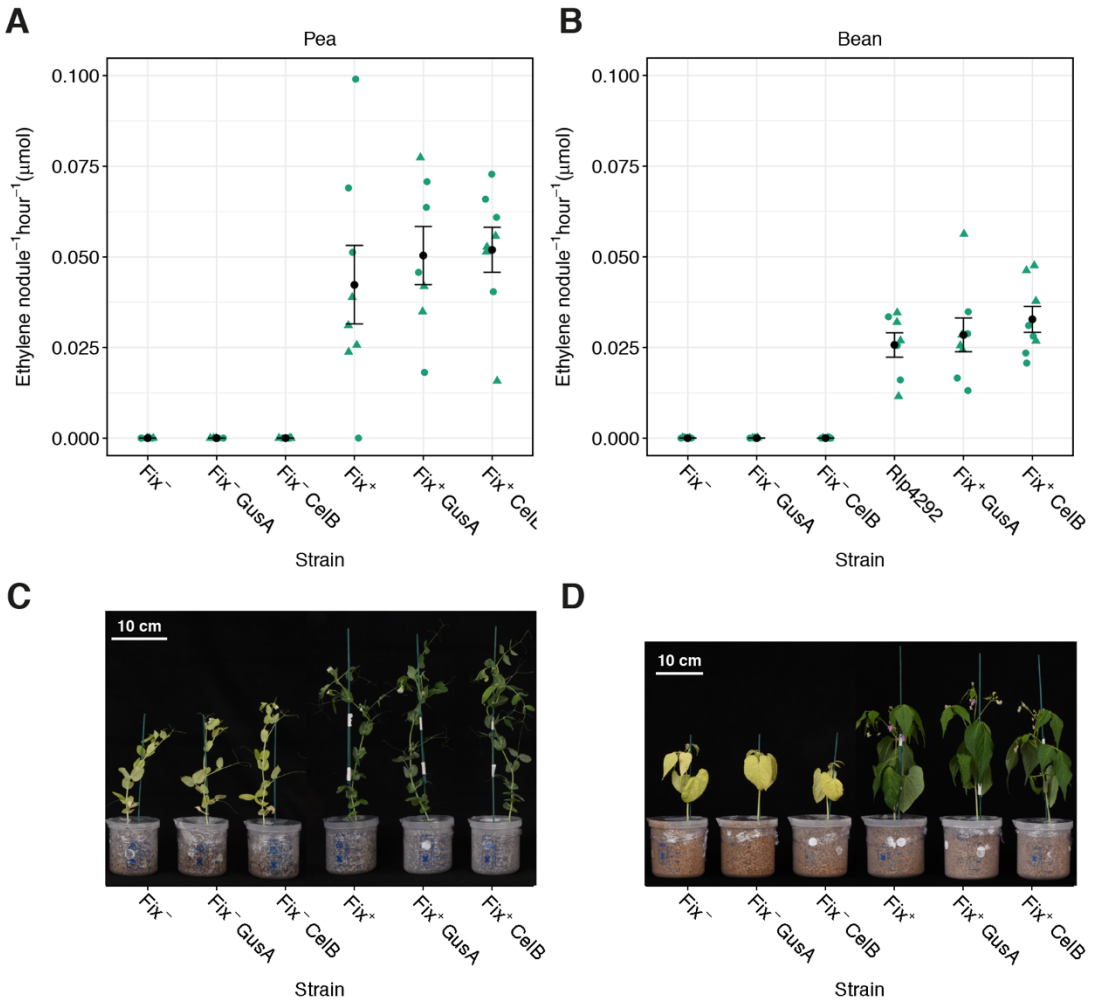


Figure 5.8 – The effect of *gusA* and *celB* marker genes on nitrogen-fixation rates and plant appearance. Strains marked with *gusA* and *celB* had similar nitrogen fixation rates to their unmarked parent strains for Fix⁻ and Fix⁺ in (A) pea and (B) bean. (A, B) Points represent an average of three technical replicates per independent replicate plant. Circles and triangles indicate data from independent experiments. Black points indicate the overall mean with one standard error. (A) Pea plants were grown for 28 days, n = 7-8. (B) Bean plants were grown for 35 days, n = 7-8. Plants inoculated with the *gusA*- and *celB*-marked strains were similar in appearance to those inoculated with the unmarked parent strains for Fix⁻ and Fix⁺ in (C) pea and (D) bean. (C, D) Images shown are representative of 7-8 biological replicates.

For pea, the marker genes did not significantly affect the nitrogen fixation rate of strains (non-significant effect of marker, $F_{2,42} = 0.0905$, $p = 0.7650$), so this term was removed from the model. On average, the Fix$^+$ strains fixed 0.0481 µmol ethylene nodule^{-1} hour^{-1} (95% CI [0.0412, 0.0551], **Figure 5.8*A***), and Fix$^-$ strains fixed 0.0000 µmol ethylene nodule^{-1} hour^{-1} (95% CI [-0.0071, 0.0071], **Figure 5.8*A***). Similarly, for bean, the marker genes did not significantly affect the nitrogen fixation rate of strains (non-significant

effect of marker, $F_{2,43}$ = 0.0407, p = 0.8410). On average, the Fix$^+$ strains fixed 0.0291 μmol ethylene nodule^{-1} hour^{-1} (95% CI [0.0259, 0.0324], **Figure 5.8*B***), and Fix$^-$ strains fixed 0.0000 μmol ethylene nodule^{-1} hour^{-1} (95% CI [-0.0032, 0.0033], **Figure 5.8*B***). Plants inoculated with the *gusA*- and *celB*-marked strains were similar in appearance to those inoculated with the unmarked parent strains for Fix$^-$ and Fix$^+$ for both pea (**Figure 5.8*C***) and bean (**Figure 5.8*D***). This demonstrates that the *gusA* and *celB* marker genes did not differentially affect the nitrogen-fixation rates of any of the strains.

5.4.3.6 Nodulation ability and nodule size for *gusA*- and *celB*-marked strains

As the *gusA* and *celB* marker genes did not impact the growth rate *in vitro*, or nitrogen-fixation rate of strains, we then compared their ability to nodulate the host plant when in competition, and the sizes of these nodules. The ability of a strain to form nodules indicates both its *in vivo* growth rate, and its ability to nodulate. It was important to determine that the marker genes did not have a differential effect on the nodulation ability or nodule size of the strains, as any differences seen in competition assays should be due to differences in the parental strains (Fix$^+$ or Fix$^-$), not the marker used.

Pea plants were co-inoculated with a 1:1 ratio of *gusA*-marked Fix$^+$ (OPS2306) and *celB*-marked Fix$^+$ (OPS2307), or *gusA*-marked Fix$^-$ (OPS2308) and *celB*-marked Fix$^-$ (OPS2309). One pre-germinated pea seed was potted per 500 ml pot containing medium vermiculite and inoculated with a 1:1 mixture of strains totalling 1 x 10^4 cells per pot. Bean plants were co-inoculated with a 1:1 ratio of *gusA*-marked Fix$^+$ (OPS2373) and *celB*-marked Fix$^+$ (OPS2348), or *gusA*-marked Fix$^-$ (OPS2374) and *celB*-marked Fix$^-$ (OPS2349). Two pre-germinated bean seeds were potted per 1 L pot containing fine vermiculite and co-inoculated with a 1:1 mixture of strains totalling 1 x 10^4 cells per pot.

In two independent experiments, four replicate plants per treatment and two water controls of each host plant were grown in a fully randomised design for 28 days for pea, and 35 days for bean. After seven days plants were thinned to one per pot.

At harvest, roots were sequentially stained for GusA and CelB[135,158]. The number of nodules formed by each strain was counted, and the area of the ten largest nodules per strain per plant were measured to ensure that all selected nodules were at a similar developmental stage. Pea and bean data were analysed separately. The percentage of nodules formed by the *gusA*-marked strain was analysed as a function of the identity of the parent strain (Fix^+ and Fix^-), using a generalised linear model with a quasibinomial error distribution. The size of nodules was analysed as a mixed effects model, with fixed effects of the identity of the parent strain (Fix^+ and Fix^-), the marker gene (*gusA* and *celB*), and their interaction; and plant as a random effect, as the nodule sizes of two strains on each plant were measured.

The marker gene affected the number of nodules formed for bean, but not pea. For pea, the percentage of nodules formed by the *gusA*-marked strains did not vary with the identity of the parent strain ($F_{1,14} = 0.0319$, $p = 0.8607$), so this term was removed from the model. No effect of marker gene was observed for pea: the *gusA*-marked strains occupied a mean of 46.30% of nodules (95% CI [41.96, 50.69]), which is not significantly different from 50%, showing that *gusA*- and *celB*- marked strains formed similar numbers of nodules. In contrast, there was an effect of marker gene on the competitiveness of bean strains, which varied with the identity of the parent strain ($F_{1,12} = 10.161$, $p = 0.0078$), so this term was retained in the model. For the Fix^+ strains, the *gusA*-marked strain occupied a mean of 35.6% of nodules (95% CI [22.8, 50.7]), whilst for the Fix^- strains, the *gusA*-

marked strain occupied a mean of 70.9% of nodules (95% CI [54.4, 83.3]), which is significantly more than 50%.

Contrastingly to nodule number, the marker gene had a significant effect on nodule size for pea, but not bean. For pea, there was no significant interaction between the marker gene and the identity of the parent strain ($F_{1,14}$ = 1.8998, p = 0.1897), so this interaction term was removed from the model. However, the marker gene had a significant effect on nodule size ($F_{1,14}$ = 5.2541, p = 0.0368). *celB*-marked strains formed larger nodules than *gusA*-marked strains (0.2850 mm^2 larger, 95% CI [0.0341, 0.5358], **Figure 5.9*A***).

For bean, there was a significant interaction between the marker gene and the identity of the parent strain ($F_{1,12}$ = 5.2655, p = 0.0406), so this interaction term was retained in the model. For the Fix$^-$ strains, the *celB*-marked strains formed smaller nodules than *gusA*-marked strains, but the difference was not significant (0.8207 mm^2 smaller, 95% CI [-0.3089, 1.9504], **Figure 5.9*B***). For the Fix$^+$ strains, the *celB*-marked strains formed larger nodules than *gusA*-marked strains, but the difference was not significant (1.077 mm^2 larger, 95% CI [-0.0522, 2.2071], **Figure 5.9*B***).

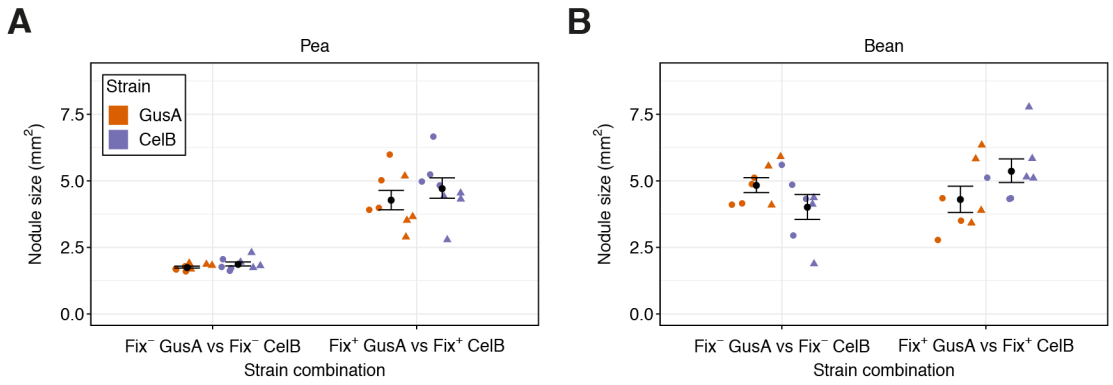


Figure 5.9 – The effect of *gusA* and *celB* marker genes on nodule size. (A) For pea, *celB*-marked strains formed larger nodules than *gusA*-marked strains for both strain combinations. Pea plants were grown for 28 days, n = 8. (B) For bean, *celB*-marked strains formed smaller nodules than *gusA*-marked strains for the Fix$^-$ strains, but larger nodules than *gusA*-marked strains for the Fix$^+$ strains. Bean plants were grown for 35 days, n = 7-8. (A, B) Points represent an average of ten technical replicates for *gusA*- and *celB*-marked nodules per independent replicate plant. Circles and triangles indicate data from independent experiments. Black points indicate the overall mean with one standard error.

Together, these results show that the *gusA* and *celB* marker genes do not affect the growth rate or nitrogen-fixation rate for pea or bean. However, nodules formed by the *celB*-marked strains are larger in pea, and in bean *celB*-marked Fix$^+$ strains form larger nodules while the *celB*-marked Fix$^-$ nodules are smaller. Additionally, while the marker gene does not affect the number of nodules formed in pea, for bean, the *gusA*-marked Fix$^-$ strain forms a significantly larger percentage of the nodules compared with the *celB*-marked Fix$^-$ strain. Given the effects of the marker gene on nodule size for pea, and on both nodule size and nodule number for bean, these effects need to be considered in analyses of nodule sizes and nodule numbers for further experiments using these strains.

5.4.4 Testing partner choice and sanctioning in pea and bean

To test for partner choice and sanctioning in determinate and indeterminate nodules, we inoculated pea and bean plants with different ratios of Fix$^+$ and Fix$^-$ strains. Partner choice was tested for by comparing the number of nodules formed by Fix$^+$ and Fix$^-$ strains when inoculated at different ratios. If partner choice is not exerted by the host, we would expect the percentage of nodules containing the Fix$^+$ strain to reflect the percentage of Fix$^+$ strain in the inoculum. Sanctioning was tested for by measuring the size of Fix$^+$ and Fix$^-$ nodules, with smaller Fix$^-$ nodules indicating sanctioning.

Pea plants were co-inoculated with a 10:1, 1:1, or 1:10 ratio of *gusA*-marked Fix$^+$ (OPS2306) and *celB*-marked Fix$^-$ (OPS2309), or *gusA*-marked Fix$^-$ (OPS2308) and *celB*-marked Fix$^+$ (OPS2307) at ratios of 10:1, 1:1, or 1:10. One pre-germinated pea seed was potted per 500 ml pot containing medium vermiculite and inoculated with a mixture of strains totalling 1 x 10^4 cells per pot. Bean plants were co-inoculated with a 10:1, 1:1, or 1:10 ratio of *gusA*-marked Fix$^+$ (OPS2373) and *celB*-marked Fix$^-$ (OPS2349), or *gusA*-

marked Fix$^-$ (OPS2374) and *celB*-marked Fix$^+$ (OPS2348). Two pre-germinated bean seeds were potted per 1 L pot containing fine vermiculite and inoculated with a mixture of strains totalling 1 x 10^4 cells per pot. In two independent experiments, two replicate plants per treatment and two water controls per host plant were grown in a fully randomised design, 28 days for pea and 35 days for bean. After seven days plants were thinned to one per pot.

At harvest, roots were sequentially stained for GusA and CelB[135,158]. The number of nodules formed by each strain was counted. The percentage of nodules formed by the Fix$^+$ strain was analysed separately for pea and bean, with a linear model as a function of the identity of the parent strain (Fix$^+$ and Fix$^-$), the marker gene (*gusA* and *celB*), and their interaction. If plants did not exert partner choice, we expected a 1:1 ratio between the percentage of Fix$^+$ strain in the inoculum and the percentage of nodules which are Fix$^+$, represented by a slope of 1 which passes through the origin.

In addition to nodule counts, the area of the five largest nodules per strain per plant was measured to ensure that all selected nodules were at a similar developmental stage. Five nodules were measured rather than ten, as some plants did not have ten nodules of each type due to the range of inoculation ratios used. The size of nodules was analysed separately for pea and bean as a mixed effects model, with fixed effects of the identity of the parent strain (Fix$^+$ and Fix$^-$), the marker gene (*gusA* and *celB*), and their interaction; and plant as a random effect, as the nodule sizes of two strains on each plant were measured. The size of the five largest Fix$^-$ nodules in 1:1 co-inoculation was compared to the sizes of the five largest in single inoculation from **Figure 5.9** using a two-sample t-test, as these experiments were conducted at the same time. Fix$^-$ nodules which were

smaller than Fix$^+$ nodules would indicate sanctioning, especially if Fix$^-$ nodules were smaller when competing against Fix$^+$, compared to when inoculated alone.

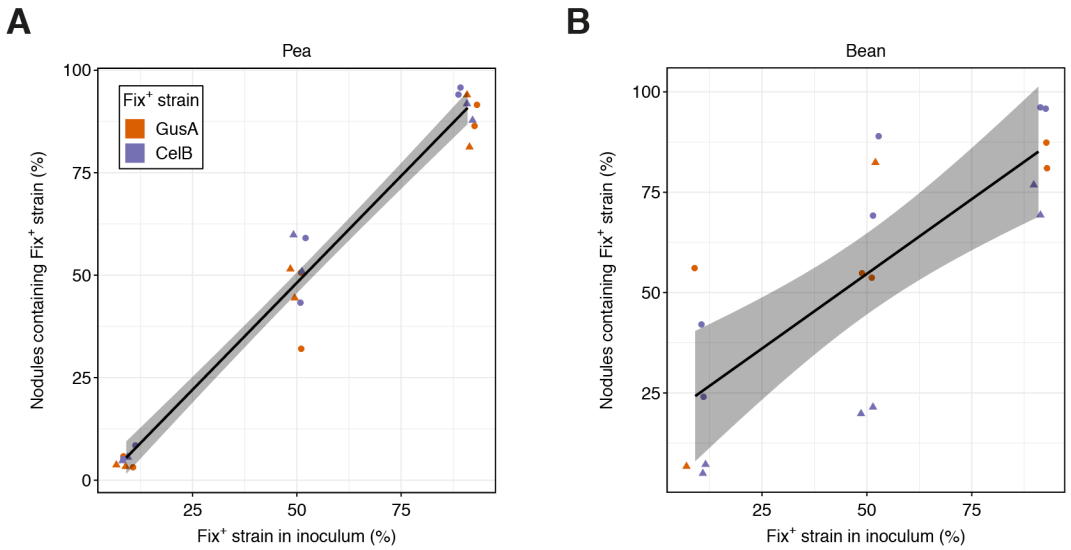


Figure 5.10 – Partner choice in pea and bean. For (A) pea and (B) bean the percentage of nodules containing the Fix$^+$ strain was the same as the percentage of Fix$^+$ strain in the inoculum. (A) Pea plants were grown for 28 days, n = 8. (B) Bean plants were grown for 35 days, n = 6-8. (A, B) Points represent an average of three technical replicates per independent replicate plant. Circles and triangles indicate data from independent experiments. The slope of the regression is indicated by the black line, with the 95% CI indicated by the grey area.

For both pea and bean, the percentage of nodules containing the Fix$^+$ strain was the same as the percentage of Fix$^+$ strain in the inoculum. For pea, the effect of marker gene did not vary with the inoculation ratio (no significant interaction, $F_{1,20} = 0.1291$, $p = 0.7231$), so this interaction term was removed from the model. The resultant slope of the regression was 1.0428, 95% CI [0.9738, 1.1119], which is not significantly different from 1 ($t_{22} = 10.755$, $p = 0.0090$). However, the marker gene did impact the percentage of nodules formed by Fix$^+$ ($F_{1,21} = 4.8611$, $p = 0.0388$). Additionally, the intercept for the *celB*-marked strains was -1.5546 (95% CI [-6.3031, 3.1938]), which is not significantly different from zero ($t_{22} = -0.6809$, $p = 0.5031$). However, the intercept for the *gusA*-marked strains was -6.4439 (95% CI [-11.1923, -1.6955]), which is significantly less than

zero (t_{22} = -2.8222, p = 0.0099), showing that *gusA*-marked Fix$^+$ strains form slightly fewer nodules than expected given the percentage of Fix$^+$ in the inoculum (**Figure 5.10*A***).

For bean, the effect of marker gene did not vary with the inoculation ratio (no significant interaction, $F_{1,15}$ = 0.2130, p = 0.6510), so this interaction term was removed from the model. The resultant slope of the regression was 0.7443 (95% CI [0.4343, 1.0543]), which is not significantly different from 1 (t_{17} = -1.7408, p = 0.0998). Additionally, the marker gene ($F_{1,16}$ = 0.8124, p = 0.3808) did not affect the percentage of nodules formed by Fix$^+$. The intercept of the line was 17.4536 (95% CI [-1.0355, 35.942699]), which is not significantly different from zero (t_{17} = 1.9916, p = 1.0000) (**Figure 5.10*B***).

For both pea and bean, the Fix$^+$ strain formed larger nodules than the Fix$^-$ strain for all inoculation ratios. This difference was significant when either the five largest nodules were measured (pea difference of 7.0678 mm^2, 95% CI [5.9286, 8.2069], **Figure 5.11*A***; bean difference of 3.4866 mm^2, 95% CI [2.4424, 4.5309], **Figure 5.11*B***), or the three largest nodules were measured (pea difference of 8.1110 mm^2, 95% CI [5.5721, 10.6491] **Figure 5.11*C***; bean difference of 3.7185 mm^2, 95% CI [2.5921, 4.8450], **Figure 5.11*D***). Additionally, for both nodule types, the Fix$^-$ strain formed smaller nodules when co-inoculated with Fix$^+$, compared to when inoculated alone. For the largest five pea nodules the difference was 0.4086 mm^2, 95% CI [0.1248, 0.6924] (t_3 = 10.755, p = 0.0090). For the largest five bean nodules the difference was 1.3088 mm^2, 95% CI [0.4921, 2.1255] (t_3 = 18.647, p = 0.0034).

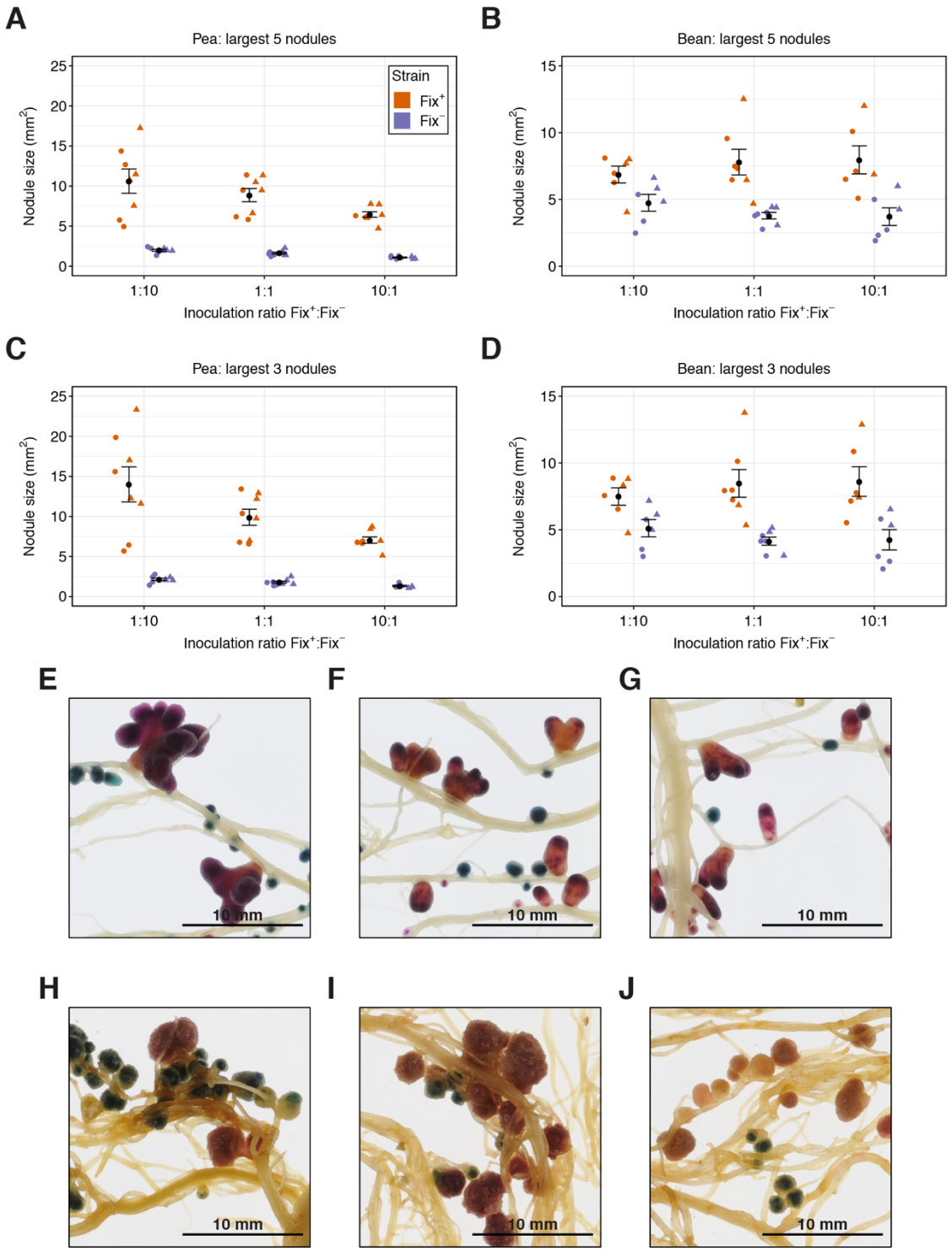


Figure 5.11 – Sanctioning in pea and bean. (A-D) For both pea and bean, Fix$^+$ formed larger nodules than Fix$^-$ at all inoculation ratios. (C) When only the largest three nodules were measured, the difference in size between Fix$^+$ and Fix$^-$ nodules for pea was largest when Fix$^+$ nodules were rare. (E-J) Fix$^+$ nodules (in this case *gusA*-marked, magenta) were large when competing against Fix$^-$ (*celB*-marked, blue) for pea at a (E) 1:10, (F) 1:1, and (G) 10:1 ratios; and for bean at a (H) 1:10, (I) 1:1, and (J) 10:1 ratios. (A, C, E-G) Pea plants were grown for 28 days, n = 8. (B, D, H-J) Bean plants were grown for 35 days, n = 6-8. Points represent an average of (A, B) five or (C, D) three technical replicates for *gusA*- and *celB*-marked nodules per independent replicate plant. Circles and triangles indicate data from independent experiments. Black points indicate the overall mean with one standard error. (E-J) Scale bar indicates 10 mm, images shown are representative of 6-8 biological replicates.

For bean, the scale of this size difference was not dependent on the inoculation ratio when either five or three nodules were measured (no significant interaction between the identity of the parent strain and the inoculation ratio, five nodules $F_{2,16} = 1.7787$, $p = 0.2007$; three nodules $F_{2,16} = 1.3782$, $p = 0.2804$, **Figure 5.11*B, D***). This shows that the size difference was consistent across different inoculation ratios. Contrastingly, for pea, the size difference was larger when the Fix$^+$ strain formed a smaller percentage of the inoculation ratio, and therefore Fix$^+$ nodules were rare. However, this effect could only be detected when the largest three nodules were measured, and not the largest five nodules (no significant interaction between the identity of the parent strain and the inoculation ratio for five nodules $F_{2,41} = 2.6518$, $p = 0.0826$; significant interaction for three nodules $F_{2,41} = 5.0439$, $p = 0.0110$, **Figure 5.11*A, C***).

Together, these results suggest that both beans, with determinate nodules, and peas, with indeterminate nodules, can sanction ineffective strains, but do not preferentially form nodules with more effective strains. Neither host plant formed more nodules with the Fix$^+$ strain than would be expected given the inoculation ratio. In terms of nodule size, Fix$^+$ formed larger nodules than Fix$^-$ strains for both pea and bean when in co-inoculation (**Figure 5.11**), and Fix$^-$ nodules were smaller when co-inoculated with Fix$^+$ than when they were inoculated alone. These size differences suggest that both host plants can sanction nodules containing Fix$^-$ strains when a better strain is available. Furthermore, for pea, the Fix$^+$ strains are largest when they are rare (**Figure 5.11*C***), suggesting that the plant allocates even more resources to these beneficial nodules when there are only a few nodules available.

5.5 Discussion

In this chapter we demonstrated that host plants with either determinate or indeterminate nodules do not preferentially form nodules with rhizobia which fix more nitrogen, but do sanction nodules containing strains which do not fix nitrogen. We tested this by inoculating bean (determinate nodules) and pea (indeterminate nodules) with different ratios of otherwise isogenic fixing and non-fixing strains. We also marked and tested these strains with chromogenic markers to allow nodules of different strains to be distinguished. When both fixing and non-fixing strains are available, we showed that for both pea and bean, the percentage of nodules containing the fixing strain closely reflected the percentage of fixing strain available in the inocula, showing no evidence of partner choice. However, nodules containing the non-fixing strain were smaller than nodules containing the fixing strain, and were smaller when competing against the fixing strain than when inoculated alone, indicating that the host plants were sanctioning these poorly-performing nodules when a better alternative was available.

We compared between determinate and indeterminate nodules using strains RlvA34 (pea) and Rlp4292 (bean), which share the same chromosome, but have different Sym plasmids, meaning they form nodules with different hosts. This system allows a powerful comparison as these strains are as similar as possible. This means that any differences we see between the response of determinate and indeterminate hosts is likely due to differences between the nodule types, and not the confounding effect of using two very different strains which inoculate hosts with different nodule types.

We generated otherwise isogenic non-fixing versions of these strains by inserting a ΩSp^r cassette in the *nifK* gene of both strains. We confirmed that these strains were non-fixing

and that their hosts had a similar shoot dry biomass to uninoculated control plants, showing that these strains gave no benefit to their host plants. Additionally, in single inoculations the non-fixing strain formed more nodules than the fixing strain, as commonly seen when host plants are nodulated with non-fixing strains[107,108] due to the autoregulation of nodulation[116,201]. The comparison of otherwise isogenic fixing and non-fixing strains is an improvement on previous studies where naturally-occurring strains of rhizobia have been used[77,89,91,94,95,98,99], as we can determine the host response to the nitrogen fixation abilities of its rhizobial partners, rather than confounding this with the host response to other naturally-differing strain traits.

To determine the host response to fixing and non-fixing strains, we inoculated the host with both of these strains in combination. To distinguish between nodules formed by the two strains, strains were marked with GusA and CelB chromogenic markers. At harvest, the rooting systems underwent a staining process which rendered nodules containing marked strains either magenta and blue[135,158], allowing nodules of the two strains to be counted and measured. We initially attempted a plasmid-based marker system, as successfully used in a range of hosts including RlvA34 and Rlp4292[108,113,144], however plants inoculated with plasmid-marked strains had small, chlorotic and non-fixing nodules. We suspected this was due to an incompatibility between the host strain and either the RK2 replicon or neomycin resistance cassette in the plasmid, but due to time pressures we did not further investigate this. Instead, to avoid any potential issues with plasmid incompatibility we marked strains chromosomally with mini-Tn*7* markers[149,151] as in **Chapter 3**. We demonstrated that strains with these markers were able to nodulate plants, that their host plants looked healthy, and that the marker genes did not affect the nitrogen-fixation ability or the growth of the rhizobia.

We then tested whether the two marker genes affect the number or size of nodules formed by the marked strains when in co-inoculation, to simulate the co-inoculation which would be used to test for partner choice and sanctioning. For pea, the *celB*-marked strains formed larger nodules than the respective *gusA*-marked strains when in co-inoculation. For bean, the *celB*-marked Fix$^-$ strain forms fewer nodules than the *gusA*-marked Fix$^-$ strain when in co-inoculation.

The observed effects of marker gene on nodule numbers and sizes may be due to human error in the manual counting and measurement of these nodules. To adjust for this, nodules were counted three times, and the average of these counts was used in the analysis. This method was shown to be robust, as in **Chapter 3**, nodules were counted in the same manner when comparing fluorescent markers and there was no detected difference in the nodulation ability of these strains. It is also possible that the variance in numbers was due to difficulty in distinguishing between individual nodules. This is particularly difficult in bean, where nodules are clustered close together and the thin veins running across their surface can be confused with nodule edges. To reduce any effects of human bias, the automation of the identification, counting, and measurement of nodules using a machine learning tool was attempted using the Trainable Weka Segmentation macro for ImageJ[202]. However, this was not taken further as the programme struggled to distinguish magenta *gusA*-marked nodules from the rooting system. Additionally, the tool struggled to differentiate between nodules when close together, particularly on bean, and therefore we decided that manual measurements gave the best possible results for this work, especially as any biases are likely to effect nodules of both types equally. The identity of the marker gene was included in the analyses of the partner choice and sanctioning experiments to account for any effects of the gene on nodule size or number.

We then showed that both pea and bean do not exert partner choice on strains varying in their nitrogen-fixation ability. Specifically, we showed that the percentage of nodules formed by the fixing strain was the same as the percentage of fixing strain applied in the inoculum. We included the effect of the marker gene in these strains, and found no effect of marker gene in bean. In pea, *gusA*-marked strains formed fewer nodules than expected, but as there was no interaction between the marker gene and the inoculum ratio, the percentage of nodules formed by the fixing strain was still proportional to the percentage of fixing strain in the inoculum. This complements work showing that the competitiveness of naturally occurring strains to form nodules and their ability to fix nitrogen are independent traits[203,204]. This makes sense, as there are many other factors which may vary in naturally occurring strains and affect their ability to form nodules[49,91,100–102]. We considered only the host response to a mutation occurring only in a nitrogen-fixation gene, as may reasonably happen to a strain in the field enabling it to cheat. Our results suggest that hosts with either nodule type are not able to detect a difference between strains at the nodulation stage, and therefore cannot preferentially form nodules with a fixing strain over a non-fixing strain, in line with other studies in indeterminate hosts[105,107].

We also demonstrated that both pea and bean are able to sanction poorly performing strains, as nodules containing a non-fixing strain were significantly smaller than those containing a fixing strain, in line with findings from other studies[88,90,93,105,106,108]. We also showed that the non-fixing strain forms smaller nodules when co-inoculated with the fixing strain than when inoculated alone. This suggests that when a host plant has a better strain available, it reduces resource allocation to a non-fixing strain at an earlier stage

than if only a non-fixing strain was available, reducing the development of these poorly-performing nodules.

Interestingly, while the host response to non-fixing strains was similar for both nodule types in terms of nodule size, we did see a difference in the response to fixing strains. In pea, we saw that fixing nodules were considerably larger when rare (when the fixing strain formed a smaller percentage of the inoculum) compared to when common. This suggests that the allocation of resources to nodules also depends on the systemic nitrogen status of the plant. When the plant has few fixing nodules, so a low systemic nitrogen status, they can allocate more resources to each of these nodules allowing further development compared to when these nodules are common. The fact that we saw this size difference only in pea and not in bean could be explained by determinate nodules lacking a persistent meristem, meaning these nodules do cease growing at some point[55]. Bean may still be able to modulate resource allocation, but we could not demonstrate this by observing nodule size alone.

Our findings suggest that not only do hosts with either determinate or indeterminate nodules reduce resource allocation to non-fixing nodules, but indeterminate hosts can actively increase resource allocation to fixing nodules when rare. This provides further evidence for a systemic mechanism in host plants to compare the effectiveness of different strains and adjust resource allocation accordingly[108]. Further evidence of this mechanism could be provided by comparing the fitness and carbon transport to strains within nodules as in **Chapter 3**, and how the number of bacteroids are affected as in **Chapter 4**. Additionally, it would be interesting to compare between both nodule types as in **Chapter 4** to determine if both allow discrimination between strains sharing a

singular, mixed nodule, for which some evidence exists for indeterminate[107] and determinate[96] hosts. Finally, while here we have looked at the two main nodule types, it is unclear how universal these systems will be across the wide range of host plants and rhizobia which engage in this symbiosis.

Overall, we have demonstrated that neither pea nor bean plants can preferentially form nodules with a fixing strain, but both can sanction strains which do not fix nitrogen, despite the differences between these two types of nodule. This could aid hosts with either nodule type by preventing them wasting resources on non-fixing strains, though it is unclear how generally these findings apply across all hosts which engage in this symbiosis. Furthermore, we showed that a host with indeterminate nodules can allocate more resources to fixing nodules when they are rare, resulting in even larger nodules, giving it even more control over its symbiotic partners. Further work investigating the fitness of strains within both determinate and indeterminate nodules could determine whether sanctioning has the additional benefit of controlling the spread of ineffective strains through the population.

Chapter 6 General discussion and future perspectives

6.1 General overview

In this thesis, we aimed to further characterise the ways in which host plants can regulate their symbiosis with nitrogen-fixing rhizobia. Specifically, we investigated how host plants respond to rhizobial strains which provide little or no nitrogen. We expanded this work to include two host species: pea (*Pisum sativum)* and bean (*Phaseolus vulgaris*), which have different nodule types: indeterminate and determinate. We showed that sanctioning, rather than partner choice, is seen both hosts, and that the indeterminate host (pea) can conditionally sanction strains by comparing their nitrogen output to other available strains. These results indicate that host plants have remarkable control over their symbiotic partners.

6.2 Summary of results

6.2.1 Chapter 3: Conditional sanctioning of an intermediate fixer

In **Chapter 3**, we demonstrated that pea, which forms indeterminate nodules, can conditionally sanction an intermediate fixer. This means that the plant can make conditional decisions about whether to sanction a particular rhizobial strain: when an intermediate fixer was the best available strain, it was provided with resources by the plant; but when a better strain was available, nodules containing the intermediate fixer were sanctioned. We tested this using pea plants inoculated with different combinations of fixing, intermediate-fixing, and non-fixing derivatives of the standard laboratory rhizobial strain Rlv3841, which were otherwise isogenic. Nodules containing the intermediate fixer were large and healthy when the only alternative was a non-fixer. However, when a more effective strain was available, plants downregulated carbon transport to the intermediate fixer, and nodules containing the intermediate fixer were smaller, with reduced nitrogen fixation. Crucially, at a later stage of development, these

nodules also contained fewer reproductive rhizobia, and so would have reduced fitness, thus justifying the word 'sanctioning', which has sometimes caused controversy in the literature. Taken together, these results provide strong evidence that legumes can make conditional decisions, allowing them to regulate their interactions with symbiotic partners.

6.2.2 Chapter 4: Quantifying sanctioning at a single-cell level using flow cytometry

In **Chapter 4**, we optimised the use of flow cytometry to demonstrate that sanctioning affects all rhizobial cells within indeterminate nodules. Sanctioned nodules had fewer bacteria and bacteroids, and there was some evidence that sanctioning could discriminate between strains within a mixed nodule that contained more than one strain which differed in their nitrogen-fixation abilities. We tested this using the same host (pea) and rhizobial strains as in **Chapter 3**. Non-fixing single-strain nodules had fewer bacteroids, regardless of whether a better alternative strain was present. However, single-strain nodules containing an intermediate fixer had fewer bacteroids only when a better alternative was available for the host. This shows that the host plant can conditionally control nodule development, and therefore the number of bacteroids, depending on which strains are available. In mixed nodules, the non-fixing strain had fewer bacteria and bacteroids per mixed nodule than a fixing strain, regardless of the fixation ability of the cohabiting strain. The intermediate fixer formed a smaller proportion of the bacteria and bacteroids when sharing a nodule with the fixing strain. However, when we analysed the absolute numbers of Fix^{int} cells per nodule, rather than proportions, we found no difference between the number of Fix^{int} cells depending on its cohabiting strain. Therefore, there is no evidence from our work that an intermediate or non-fixer could prosper via the mixed nodule route,

as in order to do so, they would have to gain a fitness advantage when sharing nodules with the fixing strain, and this was not observed in any of our experiments.

6.2.3 Chapter 5: Partner choice and sanctioning in determinate and indeterminate nodules

In **Chapter 5**, we directly compared pea, which forms indeterminate nodules, with bean, which forms determinate nodules. For strains which differed only in their nitrogen fixation, we showed that the mechanism of host regulation in both plants was sanctioning (reducing resource allocation), rather than partner choice (forming fewer nodules). The novelty of this work was to use fixing and non-fixing strains of *R. leguminosarum* which were otherwise isogenic, but contained different symbiosis plasmids, allowing them to nodulate different host plants. Neither host preferentially formed nodules with the fixing strain. However, for both hosts, non-fixing nodules were smaller than fixing nodules, indicating sanctioning. Additionally, non-fixing nodules from plants co-inoculated with the fixing strain were smaller than non-fixing nodules from plants that were only inoculated with the non-fixer. This indicates that the host can compare its nodules and alter provision of resources accordingly, as the non-fixer did worse when the plant had a better option available. Furthermore, in bean, the fixing nodules grew larger as the number of fixing nodules per plant declined, which again indicates that the host can track its overall nitrogen status and alter provision to nodules accordingly. This work opens further avenues of investigation for the mechanisms of sanctioning in both nodule types.

6.3 Implications

6.3.1 Host regulation of the symbiosis

In the legume-rhizobia symbiosis, the host plants face an investment dilemma, as they usually house multiple strains of rhizobia[78–81] that can vary in their nitrogen-fixation ability[27,75,76,177]. Supporting these rhizobia is energetically costly for the host plant[14,198]. Therefore, if hosts could regulate the symbiosis by forming fewer nodules with poorly-fixing rhizobia[77,89,91,94,95,98,99], or by sanctioning poorly-fixing rhizobia[90,91,93,95,105–107], then fewer resources would be wasted on rhizobia that provide little or no nitrogen[84,108].

In **Chapter 5** we showed that for strains differing only in their nitrogen-fixation ability, the percentage of nodules containing the fixing strain closely reflected the percentage of the fixing strain in the inoculum for both the determinate (bean) and the indeterminate (pea) host. This result confirms that neither host can exert partner choice, in line with other studies using indeterminate hosts[105,107]. This makes sense, as it seems unfeasible that a plant could detect how much nitrogen a strain will provide until it forms a nodule and begins to express genes involved with nitrogen fixation[55,57,58]. This is not to say that hosts cannot chose which partners they interact with. Hosts have been observed to form fewer nodules with less beneficial strains[77,89,91,94,95,98,99], but it is not clear if this is through partner choice, or as a result of one strain's ability to form more nodules than other strains[49,91,100–102].

There are many genes required for successful nodulation of the host which are not directly related to nitrogen fixation[104] but could still affect the choice of the host plant. This is supported by research from Bourion *et al.* (2018) in pea comparing five rhizobial strains, showing that competitiveness and nitrogen-fixation ability were independent traits[204].

Therefore, just because a strain has the correct signalling molecules, does not mean it will be an effective nitrogen fixer. However, it is likely that competitiveness and nitrogen-fixation ability are somewhat linked, as there is a strong selection pressure for strains to have both traits, and genes for these traits are often found together on Sym plasmids[104]. This could explain why research, also carried out in pea by Mendoza-Suárez *et al.* (2020) that compared 84 strains of rhizobia, showed that the most competitive strains were the most effective[113]. Therefore, while partner choice is important for selecting likely beneficial partners, the ability to discriminate between strains based on their nitrogen-fixation ability alone is also important for the host.

The results from **Chapter 5** showed that both pea and bean could discriminate between strains based on their nitrogen-fixation ability, and sanction non-fixing nodules. From these results, and previous studies of sanctioning[90,93,94,105–107], it is impossible to determine whether the response to a particular strain is simply proportional to its fixation ability, or whether the plant can compare nodules and invest in the best strain possible (conditional sanctioning). However, the results from **Chapter 3** and **Chapter 4** confirm that plants can conditionally sanction nodules, as they sanctioned an intermediate fixer only when a better option was available[108]. This shows that the plant must have a mechanism to compare the nitrogen status of its nodules (or strains within nodules) and invest only in the most beneficial strains in order to meet its nitrogen requirements.

Additionally, in **Chapter 5**, we showed that in peas, the fixing nodules were larger as the number of fixing nodules per plant declined. This shows that the plant can 'reward' high-performing nodules when rare. This supports the idea that the plant must be able to compare the nitrogen status of nodules to its own systemic nitrogen status[108] and

provision accordingly. This presumably results in more resources being supplied to fixing nodules when they are rare, explaining their larger size. Further evidence that plants have the ability to compare nodule nitrogen status to their systemic nitrogen status is provided by split root experiments. In such experiments, hosts provided with external nitrate on one half of the split root were seen to sanction fixing nodules on the other split-root half, presumably because they were no longer required[119].

Taken together, there is now clear evidence of host regulation at a whole-nodule level, which is even more sophisticated than previously thought. However, mixed nodules can also form, where the nodule is occupied by more than one rhizobial strain[96,110–113]. There is currently only limited evidence[96,107] that host plants can discriminate and sanction poorly-performing strains within a mixed nodule. **Chapter 4** provides further evidence for sanctioning of both a non-fixer and an intermediate fixer within a mixed nodule, but this evidence was not conclusive. Therefore, further work needs to be carried out in this area.

In summary, the results from this thesis support the hypothesis that at least some legume plants are able to compare the nitrogen status of each nodule with their systemic nitrogen status and provision nodules accordingly, allowing them to make flexible, conditional decisions that will optimise growth. However, whether this cue can act within a mixed nodule remains unclear.

6.3.2 Evolutionary impacts of host regulation

Sanctioning can be selected for at an individual plant level if the benefits of sanctioning (reduced waste of resources) outweigh the costs. However, sanctioning also reduces the

fitness of poorly-fixing rhizobia and may prevent their spread throughout the soil population[74,77,90], and could ultimately prevent the breakdown of the symbiosis over evolutionary time[85,86].

As rhizobia are not vertically transmitted, they must instead be taken up from the soil by each host plant, providing an opportunity for a poorly-fixing strain to form nodules[77]. If the host did not sanction these poorly-performing strains, these strains could leave the costly process of nitrogen fixation[82] to other strains, and gain higher fitness through investing more in reproduction[83,84].

The ability of sanctioning to prevent the spread of poorly-fixing strains depends on the reduction in fitness that they experience in sanctioned nodules, which may differ depending on the nodule type. In **Chapter 5**, we demonstrated reduced nodule size in both determinate and indeterminate non-fixing nodules; however, we did not directly quantify the number of reproductive rhizobia, which may differ between the nodule types. Additionally, the results in **Chapter 4** show that at 28 dpi, non-fixing nodules had fewer bacteroids, but there was no reduction in bacteria until 56 dpi. In fact, at 28 dpi, non-fixing nodules actually had more bacteria per nodule than fixing nodules. This supports results from Daubech *et al*. (2017), who also only observed a reduced fitness of a non-fixing strain at a later time point in nodule development[107]. Accounting for this delay in the effect of sanctioning is important, as subsequent modelling and empirical results from Daubech *et al*. (2017) demonstrated that a non-fixing strain is more likely to be eradicated from a population over nodulation cycles which are long enough to reduce the fitness of non-fixing strains[107].

Secondly, the ability of sanctioning to prevent the spread of poorly-fixing strains also depends on whether plants can sanction poorly-fixing strains within mixed nodules, or if mixed nodules offer a way for poorly-fixing strains to prosper[97]. If plants cannot sanction poor fixers within mixed nodules, then a poor fixer that cohabits with a fixing strain may gain higher fitness through investing more resources in reproduction[83,84]. In **Chapter 5** we provide some evidence that pea can sanction poor fixers within mixed nodules, as intermediate fixers form a smaller proportion of bacteria and bacteroids when sharing a nodule with a fixing strain, and non-fixers a smaller proportion of bacteroids, which complements work in determinate[96] hosts. However, our evidence was not conclusive, because the absolute numbers of intermediate-fixing bacteria and CFUs, and non-fixing CFUs, was not decreased when sharing a nodule with a fixing strain, as has been previously observed[107]. It has been argued that the effectiveness of sanctions to maintain cooperation in symbioses depends on the ability of the host to compartmentalise its symbionts[115]. Therefore, the ability of the host to compartmentalise and sanction strains within a mixed nodule requires further investigation to understand the impact of mixed nodules on an evolutionary timescale.

The relevance of mixed nodules as a mechanism for poor fixers to spread depends, among other things, on their relative frequency, which varies with the host plant[77,91,97,107,112,113] and the inoculation density[105]. Even if poor fixers could gain a fitness advantage within mixed nodules (which we did not observe), this probably wouldn't allow poor fixers to persist unless many mixed nodules could be formed. In our experiments, only a handful of mixed nodules were formed per plant, compared to a few hundred single-strain nodules. This would make mixed nodules an unlikely route to success, as even if a fitness benefit is gained in these nodules, the population increase of poor fixers through the few

mixed nodules would not be as high as the population increase of good fixers through the many single-infection nodules. However in other species, such as *Lotus strigosus*[91] and *Glycine max*[112], half or more nodules were found to have mixed occupancy. Here mixed nodules as a route to success for poor fixers is more feasible, as the poor fixer can increase its population size through a greater number of nodules.

Finally, sanctioning can only prevent the spread of poorly-fixing strains if this actually affects the number of reproductive rhizobia released back into the soil once the nodule senesces. Modelling and empirical work by Daubech *et al*. (2017) suggests that a fixing strain could eradicate a non-fixing strain over several nodulation cycles due to the effect of host sanctions[107]. In this multi-generational experiment, nodules were collected and crushed to use as an inoculum for the next generation, but it is not clear whether the same number of viable rhizobia would be released from the nodule naturally when the nodule senesces. Additionally, the time point at which rhizobia are released would also matter. In **Chapter 4** we saw that non-fixing strains often had more bacteria or CFUs per nodule or mixed nodule than their fixing counterparts at 28 dpi, but fewer at 56 dpi. Therefore, the relative numbers of rhizobia which are released into the soil would depend on whether this occurs at 28 dpi, or after 56 dpi.

Additionally, rhizobial survival in the soil may be affected by the production of rhizopines by bacteroids, which are secreted into the soil and can be used as a carbon source[205–207]. It is possible that sanctioned strains could still benefit from the rhizopines produced by non-sanctioned strains, though the role of rhizopines as an energy source is not fully understood[205]. Further work investigating how many viable rhizobia are released from

nodules and how they survive in the soil is essential[97,208] and would improve our understanding of the long-term dynamics of sanctioning on rhizobial populations.

Overall, the ability of sanctioning to prevent the spread of poorly-fixing strains is dependent on the fitness effects of sanctioning in single-strain nodules, the frequency of mixed nodules, and the relative fitness of different strains within mixed nodules. These values may vary widely between hosts, so a general picture of the impact of sanctioning on the symbiosis at an evolutionary time scale will require studying multiple species, beyond the handful that are normally used.

6.4 Future directions

The results in this thesis showed that hosts can conditionally sanction, or reward, rhizobial strains based on their nitrogen-fixation abilities compared to other available strains. However, further work could elucidate if strains can be sanctioned when they share a nodule, how widespread this phenomenon is, and the mechanism by which the host compares strains.

In **Chapter 4** we investigated the possibility of sanctioning within mixed nodules. We saw that the non-fixer formed a smaller proportion of bacteroids when sharing a nodule with the fixing strain, in line with previous evidence[96]. Under the same conditions the intermediate strain formed a smaller proportion of the bacteria and bacteroids, implying a fitness reduction. We did not see evidence for within-nodule sanctioning of an intermediate strain in the absolute numbers of all cells per nodule, or of the non-fixing strain in the absolute numbers of CFUs, despite previous evidence for this[107]. This may be due to the large amount of variation between biological replicates. Other results

presented in this thesis were usually averages of five or ten nodules per plant. However, the mixed-nodule results were based on a single nodule per plant, as this was the maximum number that could be identified in some cases. To increase the number, we recommend further experiments using a tenfold increase in the inoculation density: from the 10^8 cells per pot used here to 10^9 cells per pot or more, which could encourage more mixed nodules per plant[105]. This would allow results to be averaged across multiple mixed nodules per plant as in Daubech *et al*. (2017)[107], potentially reducing the variation between samples and providing a clearer conclusion of whether sanctioning can occur within mixed nodules. Further investigations of mixed nodules using the luminescent biosensors[143] as in **Chapter 3**[108] could elucidate whether the carbon supplied to different strains within a mixed nodule can differ based on their fixation ability.

In **Chapter 3** and **Chapter 4** we studied sanctioning of an intermediate fixer in pea only, which forms indeterminate nodules. It would be interesting to compare this with bean, which forms determinate nodules, as differences within the structures of the two nodule types[55] may affect how sanctioning acts. In a similar vein, this work has focused on only two host species using strains derived from the same parent, so repeating these experiments with a variety of host plants and rhizobia would elucidate how widespread this mechanism is.

In **Chapter 5** we showed that for indeterminate hosts, fixing nodules are largest when rare. Presumably this occurs because the host allocates more carbon to these nodules when rare, but use of the luminescent bioreporters[143] as in **Chapter 3**[108] could show if this is indeed the case. Acetylene reductions could also show if the increase in carbon supply upregulates nitrogen-fixation rates per nodule as in **Chapter 3**[108]. Whether the

increase in size correlates with an increase in the number of nitrogen-fixing bacteroids could be determined using flow cytometry as in **Chapter 4**. This phenomenon could also be studied using an intermediate fixer, to see whether the same effect is seen. Additionally, we only saw this size increase in indeterminate pea nodules, and not determinate bean nodules. This could be because determinate nodules do not maintain a persistent meristem and therefore cease growing at some point[55]. However, use of the methods described above would allow us to see if hosts increase carbon supply to these nodules, and if their fixation ability changes, even if their size does not.

Our results strongly suggest that pea plants can compare the nitrogen status of each nodule with some systemic nitrogen cue. However, the question remains as to what that cue might be. The cue must be able to travel over the whole root system, as split-root experiments have shown that plants can compare nodules on both halves of split roots[126], and senesce nodules on one split root half after the provision of external nitrate to the other half[119]. The cue likely also travels through the shoot, as root-shoot-root signalling has been shown to play a role in the autoregulation of nodule numbers, where the number of nodules formed is limited by the host based on its nitrate status and the number of nodules already formed[39,116–118]. A host plant mutant library could potentially be used to highlight potential candidates, by identifying hosts which cannot conditionally sanction their rhizobial partners. An example of such a library is the Tnt1-insertion mutant library of *Medicago truncatula* ecotype R108, previously used to screen for defects in nodulation and nitrogen fixation[209].

Interestingly, in **Chapter 3** we saw that a higher percentage of Fix^{int} nodules expressed P*nifH* at a later time point when competing against the Fix^{+} strain. This suggests that Fix^{int}

nodules actively increased their expression of nitrogenase genes in response to host sanctioning, by upregulating nitrogen-fixation genes in order to receive carbon from the plant. This is indicative of a biological market[210], where Fix^{int} attempts to offer more nitrogen to the host to outbid its competitor strain, as also seen in the symbiosis between plants and arbuscular mycorrhizal fungi[211]. It is not clear if the increased *nifH* expression correlates with an actual increase in nitrogen fixation, as this may be carbon-supply dependent. However, this could be further investigated by monitoring gene expression, carbon transport, and nitrogen fixation over a range of time points.

Finally, in this thesis we focused only on differences in effectiveness (the amount of nitrogen provided) of rhizobial strains. Strains may also differ in efficiency (the amount of carbon demanded per unit of nitrogen provided), and modelling suggests that hosts can maximise their growth rate by conditional sanctioning based on efficiency too[108,179]. A study of a less efficient (resource-hoarding) strain did not show evidence of sanctioning by the host plant under normal growth conditions[212] or under elevated carbon dioxide (CO_2) supply[213]. However, modelling suggests that efficiency is more important when the host is carbon-limited[108]. Therefore, it would be interesting to compare strains differing in efficiency under carbon-limited conditions, such as shading of plants to reduce photosynthesis. This could allow us to see whether the host can sanction based on efficiency in a similar way to effectiveness, or whether another signalling system could be at work.

6.5 General conclusions

This thesis shows the ability of host plants to regulate their symbiosis with rhizobial partners by sanctioning poorly performing strains. We showed that sanctioning, rather than partner choice, is seen both in indeterminate and determinate hosts, and that indeterminate hosts can conditionally sanction strains based on their nitrogen output compared to other available strains. These results indicate that host plants have remarkable control over their symbiotic partners and improve our understanding of how host plants manage interactions with multiple symbiotic partners. This of great importance when developing more effective rhizobia as alternatives to nitrogenous fertilisers.

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Appendix 1 - Supplementary data for Chapter 4

Cell type	Fluorescent marker	Mean	SE
Bacteria, 28 dpi	GFP	2.2546×10^6	2.1622×10^5
	mCherry	2.4660×10^6	3.5656×10^5
Bacteroids, 28 dpi	GFP	6.2261×10^6	5.1066×10^5
	mCherry	7.6637×10^6	8.1882×10^5

Table A1.1 – Group means and SEs for 4.4.4 Assessment of fluorescently marked strains in competition using flow cytometry (**Figure 4.5**).

Cell type	Inoculation type	Strain	Mean	SE
Bacteria, 28 dpi	Single	Fix^-	4.3812×10^6	1.4354×10^6
	Single	Fix^+	7.5713×10^5	1.8855×10^5
	Co-inoculation	Fix^-	2.2051×10^6	3.8649×10^5
	Co-inoculation	Fix^+	1.1786×10^6	3.5544×10^5
Bacteria, 56 dpi	Single	Fix^-	1.4626×10^6	5.0838×10^5
	Single	Fix^+	5.9444×10^6	1.9816×10^6
	Co-inoculation	Fix^-	7.9809×10^5	5.0340×10^5
	Co-inoculation	Fix^+	1.0653×10^7	3.6464×10^6
Bacteroids, 28 dpi	Single	Fix^-	5.3481×10^6	1.2114×10^6
	Single	Fix^+	2.9298×10^7	4.4696×10^6
	Co-inoculation	Fix^-	2.4915×10^6	4.0518×10^5
	Co-inoculation	Fix^+	2.7103×10^7	5.6041×10^6
Bacteroids, 56 dpi	Single	Fix^-	1.1017×10^6	3.4332×10^5
	Single	Fix^+	1.1160×10^7	1.9201×10^6
	Co-inoculation	Fix^-	1.4141×10^5	3.2337×10^4
	Co-inoculation	Fix^+	1.0432×10^7	2.2479×10^6

Table A1.2 – Group means and SEs for 4.4.5 Testing the effects of sanctioning on bacteria and bacteroids of Fix^- using flow cytometry (**Figure 4.6**).

Cell type	Competing strain	Mean	SE
Fix^{int} bacteria, 28 dpi	Fix^{-}	5.5428×10^{5}	1.4785×10^{5}
	Fix^{int}	4.1474×10^{5}	1.3572×10^{5}
	Fix^{+}	7.9673×10^{5}	2.4984×10^{5}
Fix^{int} bacteria, 56 dpi	Fix^{-}	8.5562×10^{6}	1.0615×10^{6}
	Fix^{int}	4.9253×10^{6}	7.1090×10^{5}
	Fix^{+}	4.2357×10^{6}	1.1387×10^{6}
Fix^{int} bacteroids, 28 dpi	Fix^{-}	5.4001×10^{6}	1.7886×10^{6}
	Fix^{int}	3.1894×10^{6}	9.6655×10^{5}
	Fix^{+}	2.8663×10^{5}	8.9069×10^{4}
Fix^{int} bacteroids, 56 dpi	Fix^{-}	5.0485×10^{6}	1.8420×10^{6}
	Fix^{int}	1.4565×10^{6}	4.1823×10^{5}
	Fix^{+}	2.3528×10^{5}	1.0380×10^{5}

Table A1.3 – Group means and SEs for 4.4.6 Testing the effects of sanctioning on bacteria and bacteroids of Fix^{int} using flow cytometry (**Figure 4.7**).

Cell type	Mixed nodule occupants	Strain	Count mean	Count SE	% mean	% SE
CFUs, 28 dpi	GFP Fix$^-$ and mCherry Fix$^-$	GFP Fix$^-$	3.7394 x 10^6	2.6742 x 10^6	-	-
		mCherry Fix$^-$	3.3859 x 10^6	2.3659 x 10^6	51.92%	5.36%
	GFP Fix$^+$ and mCherry Fix$^+$	GFP Fix$^+$	1.5648 x 10^5	7.6246 x 10^4	-	-
		mCherry Fix$^+$	4.5162 x 10^5	1.9214 x 10^5	-	-
	GFP Fix$^+$ and mCherry Fix$^-$	GFP Fix$^+$	1.8532 x 10^5	1.2189 x 10^5	-	-
		mCherry Fix$^-$	2.8710 x 10^5	1.5390 x 10^5	60.12%	6.89%
CFUs, 56 dpi	GFP Fix$^-$ and mCherry Fix$^-$	GFP Fix$^-$	8.7985 x 10^6	2.1854 x 10^6	-	-
		mCherry Fix$^-$	1.0029 x 10^7	2.7967 x 10^6	47.76%	6.29
	GFP Fix$^+$ and mCherry Fix$^+$	GFP Fix$^+$	1.7180 x 10^7	5.0632 x 10^6	-	-
		mCherry Fix$^+$	1.0965 x 10^7	3.7083 x 10^6	-	-
	GFP Fix$^+$ and mCherry Fix$^-$	GFP Fix$^+$	1.2989 x 10^7	3.3975 x 10^6	-	-
		mCherry Fix$^-$	8.3487 x 10^6	2.1331 x 10^6	41.82%	7.32%
Bacteria, 28 dpi	GFP Fix$^-$ and mCherry Fix$^-$	GFP Fix$^-$	2.2742 x 10^6	1.1287 x 10^6	-	-
		mCherry Fix$^-$	1.5252 x 10^6	4.0060 x 10^5	47.86%	6.30%
	GFP Fix$^+$ and mCherry Fix$^+$	GFP Fix$^+$	2.6557 x 10^5	1.0653 x 10^5	-	-
		mCherry Fix$^+$	1.1318 x 10^6	2.9739 x 10^5	-	-
	GFP Fix$^+$ and mCherry Fix$^-$	GFP Fix$^+$	2.0444 x 10^5	4.5005 x 10^4	-	-
		mCherry Fix$^-$	1.2158 x 10^6	7.6601 x 10^5	71.50%	5.57%
Bacteria, 56 dpi	GFP Fix$^-$ and mCherry Fix$^-$	GFP Fix$^-$	3.4569 x 10^6	5.9601 x 10^5	-	-
		mCherry Fix$^-$	2.3056 x 10^6	1.1075 x 10^6	27.96%	8.71%
	GFP Fix$^+$ and mCherry Fix$^+$	GFP Fix$^+$	6.0509 x 10^6	7.6059 x 10^5	-	-
		mCherry Fix$^+$	2.7794 x 10^6	7.8366 x 10^5	-	-
	GFP Fix$^+$ and mCherry Fix$^-$	GFP Fix$^+$	5.1375 x 10^6	1.4269 x 10^6	-	-
		mCherry Fix$^-$	2.2742 x 10^6	1.1287 x 10^6	30.95%	6.98%
Bacteroids, 28 dpi	GFP Fix$^-$ and mCherry Fix$^-$	GFP Fix$^-$	3.0789 x 10^5	1.1524 x 10^5	-	-
		mCherry Fix$^-$	2.6533 x 10^5	7.9093 x 10^4	56.75%	4.97%
	GFP Fix$^+$ and mCherry Fix$^+$	GFP Fix$^+$	7.0620 x 10^5	1.7418 x 10^5	-	-
		mCherry Fix$^+$	2.8678 x 10^6	9.4057 x 10^5	-	-
	GFP Fix$^+$ and mCherry Fix$^-$	GFP Fix$^+$	8.3799 x 10^5	2.7589 x 10^5	-	-
		mCherry Fix$^-$	5.4573 x 10^5	1.4201 x 10^5	47.53%	5.88%
Bacteroids, 56 dpi	GFP Fix$^-$ and mCherry Fix$^-$	GFP Fix$^-$	2.1496 x 10^5	5.7541 x 10^4	-	-
		mCherry Fix$^-$	3.9750 x 10^5	2.2724 x 10^5	48.48%	8.10%
	GFP Fix$^+$ and mCherry Fix$^+$	GFP Fix$^+$	1.6382 x 10^6	3.8572 x 10^5	-	-
		mCherry Fix$^+$	9.0095 x 10^5	3.1421 x 10^5	-	-
	GFP Fix$^+$ and mCherry Fix$^-$	GFP Fix$^+$	1.3600 x 10^6	5.0551 x 10^5	-	-
		mCherry Fix$^-$	3.9867 x 10^5	9.1238 x 10^4	40.15%	6.21%

Table A1.4 – Group means and SEs for 4.4.7 Testing the effects of sanctioning within mixed nodules (**Figure 4.8, Figure 4.9**).

Cell type	Cohabiting strain	Count mean	Count SE	% mean	% SE
Fix^{int} CFUs, 28 dpi	Fix^{-}	2.1592×10^{6}	1.2235×10^{6}	31.95%	5.15%
	Fix^{int}	6.9327×10^{4}	1.5749×10^{4}	43.47%	5.30%
	Fix^{+}	4.9038×10^{5}	1.9306×10^{5}	63.73%	4.89%
Fix^{int} CFUs, 56 dpi	Fix^{-}	2.2571×10^{7}	9.3216×10^{6}	39.96%	4.82%
	Fix^{int}	2.0295×10^{7}	7.6758×10^{6}	59.26%	4.87%
	Fix^{+}	7.1600×10^{6}	2.4409×10^{6}	47.58%	5.02%
Fix^{int} bacteria, 28 dpi	Fix^{-}	3.8533×10^{5}	1.8128×10^{5}	21.02%	5.82%
	Fix^{int}	5.3528×10^{4}	1.5332×10^{4}	27.32%	6.01%
	Fix^{+}	1.3056×10^{5}	3.7837×10^{4}	27.95%	5.14%
Fix^{int} bacteria, 56 dpi	Fix^{-}	5.4458×10^{5}	1.6442×10^{5}	38.94%	5.66%
	Fix^{int}	2.2639×10^{5}	6.9429×10^{4}	20.05%	4.89%
	Fix^{+}	6.0281×10^{5}	1.7936×10^{5}	29.57%	5.68%
Fix^{int} bacteroids, 28 dpi	Fix^{-}	5.5795×10^{6}	7.2427×10^{5}	59.78%	5.74%
	Fix^{int}	5.2329×10^{6}	7.6180×10^{5}	56.92%	6.25%
	Fix^{+}	4.6751×10^{6}	1.0643×10^{6}	65.51%	5.52%
Fix^{int} bacteroids, 56 dpi	Fix^{-}	1.4239×10^{6}	4.6393×10^{5}	61.95%	6.97%
	Fix^{int}	2.2569×10^{6}	8.6482×10^{5}	43.90%	7.48%
	Fix^{+}	8.3027×10^{5}	2.7765×10^{5}	34.10%	7.61%

Table A1.5 – Group means and SEs for 4.4.8 Testing the effects of sanctioning on an intermediate fixer within mixed nodules (**Figure 4.10**, **Figure 4.11**).

Appendix 2 - Published manuscript from Chapter 3

This appendix contains the manuscript in which some of the data from **Chapter 3** of this thesis have been published:

Conditional sanctioning in a legume–*Rhizobium* mutualism

Westhoek, A., Clark, L. J., Culbert, M., Dalchau, N., Griffiths, M., Jorrin, B., Karunakaran, R., Ledermann, R., Tkacz, A., Webb, I., James, E. K., Poole, P. S. & Turnbull, L. A.

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Conditional sanctioning in a legume–*Rhizobium* mutualism

Annet Westhoek[a,b,1], Laura J. Clark[a,1], Michael Culbert[a], Neil Dalchau[c], Megan Griffiths[a], Beatriz Jorrin[a], Ramakrishnan Karunakaran[d], Raphael Ledermann[a], Andrzej Tkacz[a], Isabel Webb[a], Euan K. James[e], Philip S. Poole[a,2], and Lindsay A. Turnbull[a,2]

[a]Department of Plant Sciences, University of Oxford, OX1 3RB Oxford, United Kingdom; [b]Systems Biology Doctoral Training Centre, Doctoral Training Centre, University of Oxford, OX1 3NP Oxford, United Kingdom; [c]Biological Computation, Microsoft Research Cambridge, CB1 2FB Cambridge, United Kingdom; [d]Department of Molecular Microbiology, John Innes Centre, Norwich Research Park, NR4 7UH Norwich, United Kingdom; and [e]Ecological Sciences, The James Hutton Institute, DD2 5DA Invergowrie, United Kingdom

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Legumes are high in protein and form a valuable part of human diets due to their interaction with symbiotic nitrogen-fixing bacteria known as rhizobia. Plants house rhizobia in specialized root nodules and provide the rhizobia with carbon in return for nitrogen. However, plants usually house multiple rhizobial strains that vary in their fixation ability, so the plant faces an investment dilemma. Plants are known to sanction strains that do not fix nitrogen, but nonfixers are rare in field settings, while intermediate fixers are common. Here, we modeled how plants should respond to an intermediate fixer that was otherwise isogenic and tested model predictions using pea plants. Intermediate fixers were only tolerated when a better strain was not available. In agreement with model predictions, nodules containing the intermediate-fixing strain were large and healthy when the only alternative was a nonfixer, but nodules of the intermediate-fixing strain were small and white when the plant was coinoculated with a more effective strain. The reduction in nodule size was preceded by a lower carbon supply to the nodule even before differences in nodule size could be observed. Sanctioned nodules had reduced rates of nitrogen fixation, and in later developmental stages, sanctioned nodules contained fewer viable bacteria than nonsanctioned nodules. This indicates that legumes can make conditional decisions, most likely by comparing a local nodule-dependent cue of nitrogen output with a global cue, giving them remarkable control over their symbiotic partners.

symbiosis | legume | rhizobia | sanction | resource allocation

Plants in the legume family (Fabaceae) can access atmospheric nitrogen through an intimate relationship with nitrogen-fixing rhizobia. The plant provides carbon compounds in return for usable nitrogen but faces an investment dilemma: under field conditions, plants tend to host multiple strains (1–4), which vary in how much nitrogen they provide (5–8). In experimental settings, nodules containing strains that provide little or no nitrogen show restricted development, which reduces the fitness of the rhizobia (9–11)—commonly called sanctioning. Sanctioning is presumed to be an adaptive response, likely selected to prevent the plant wasting resources on strains that provide little nitrogen. By reducing rhizobial fitness, sanctioning may prevent the spread of ineffective strains through the population, which could otherwise be invaded by cheats (9, 12, 13).

Evidence for sanctioning mostly comes from strains that are forced to become nonfixing, either by denying them a source of atmospheric nitrogen or by knocking out key nitrogen fixation genes in otherwise isogenic strains (9–11). However, these experiments do not reveal how a plant should best allocate resources among multiple natural rhizobial strains that exhibit more subtle variation in effectiveness. Investment choices matter because nonfixing strains are probably rare in the field, while poor fixers are common (5, 14, 15). Sophisticated allocation strategies would be possible if the plant could integrate information from multiple nodules and make conditional decisions so that resource allocation to a particular strain would depend on the nature of other strains present. However, empirical results obtained so far (9–11, 16) cannot distinguish this possibility from a model in which plants simply allocate resources to each nodule in proportion to the amount of nitrogen it provides.

Here, we use mathematical modeling to make predictions about optimal plant resource allocation, which we then test experimentally using pea (*Pisum sativum* L.) and otherwise isogenic fixing (Fix^+), intermediate-fixing (Fix^{int}), and nonfixing (Fix^-) derivatives of the standard laboratory rhizobial strain *Rhizobium leguminosarum* bv. *viciae* (Rlv) 3841. The modeling revealed that plants should engage in conditional sanctioning, and experimental tests showed that resource allocation to an intermediate-fixing strain is indeed conditional on which other strains are present. The plant's response affects carbon transport to the rhizobia, the size of nodules, the rates of nitrogen fixation, and rhizobial fitness. We propose that resource allocation to nodules is regulated by a combination of local and systemic cues, which together allow the plant to compare the nitrogen output of different nodules and adjust allocation accordingly.

Significance

Legumes, like peas, beans, and lentils, are high in protein because they form associations with specialized nitrogen-fixing bacteria. Plants provide the bacteria with carbon and receive nitrogen in return, but some bacterial strains provide more nitrogen than others. We show that pea plants supply resources to strains that are poor at fixing nitrogen only if they have no other choice. If a better strain is present, resources are withheld. This indicates that legumes can make conditional decisions, giving them remarkable control over their symbiotic partners. This knowledge could allow selection of plants that are better at discriminating among strains and improve understanding of soil bacterial populations. Both could help to increase yields in agricultural settings, where poor fixers are common.

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The authors declare no competing interest.

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[1]A.W. and L.J.C. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: philip.poole@plants.ox.ac.uk or lindsay.turnbull@plants.ox.ac.uk.

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Results

Model Prediction: Optimal Plant Resource Allocation Requires Conditional Sanctioning. To establish how legumes should allocate resources in order to maximize growth rates, we modeled the growth of a plant with three compartments: shoots, roots, and nodules (Fig. 1 *A* and *B*). Building new tissue of any kind requires a fixed ratio of carbon to nitrogen so that plant growth is either carbon- or nitrogen-limited. The shoot obtains carbon via photosynthesis, while nitrogen is obtained through root uptake or rhizobial nitrogen fixation. All tissues respire carbon, which can occur at different rates in different tissues. We used the model to determine how new growth should be allocated to shoot, root, and nodules to maximize instantaneous growth rates, assuming this will maximize total final plant size and hence fitness and yield.

Analytical solutions (*SI Appendix*) reveal that plants should invest in roots or nodules but not both (we assumed well-watered conditions and ignored root functions other than nitrogen uptake) (Fig. 1 *C* and *D* and *SI Appendix*, Fig. S1 *A* and *B*). The plant should invest in nodules rather than roots when the following occurs:

$$\frac{m}{d_{nod} + \gamma - d_{leaf}} > \frac{\theta c_N}{d_{root} + \gamma - d_{leaf}}, \quad [1]$$

where d_{leaf}, d_{root}, and d_{nod} are the respiration rates of shoot, root, and nodules, respectively (mg C mg tissue^{-1} · d^{-1}), γ is the rate of carbon uptake through photosynthesis (mg C mg shoot^{-1} · d^{-1}), θc_N is the rate of root nitrogen uptake (mg N mg root^{-1} · d^{-1}), and m is the rate of nitrogen fixation (mg N mg nodule^{-1} · d^{-1}). Thus, plants are more likely to invest in nodules rather than roots when the plant is nitrogen-limited and the strain in question is both effective (amount of nitrogen provided: m) and efficient (amount of carbon demanded per unit of nitrogen fixed: $e = d_{nod}/m$). The dependency of the investment decision on root nitrogen uptake means that plants should tolerate less effective and less efficient strains when they are more nitrogen-limited.

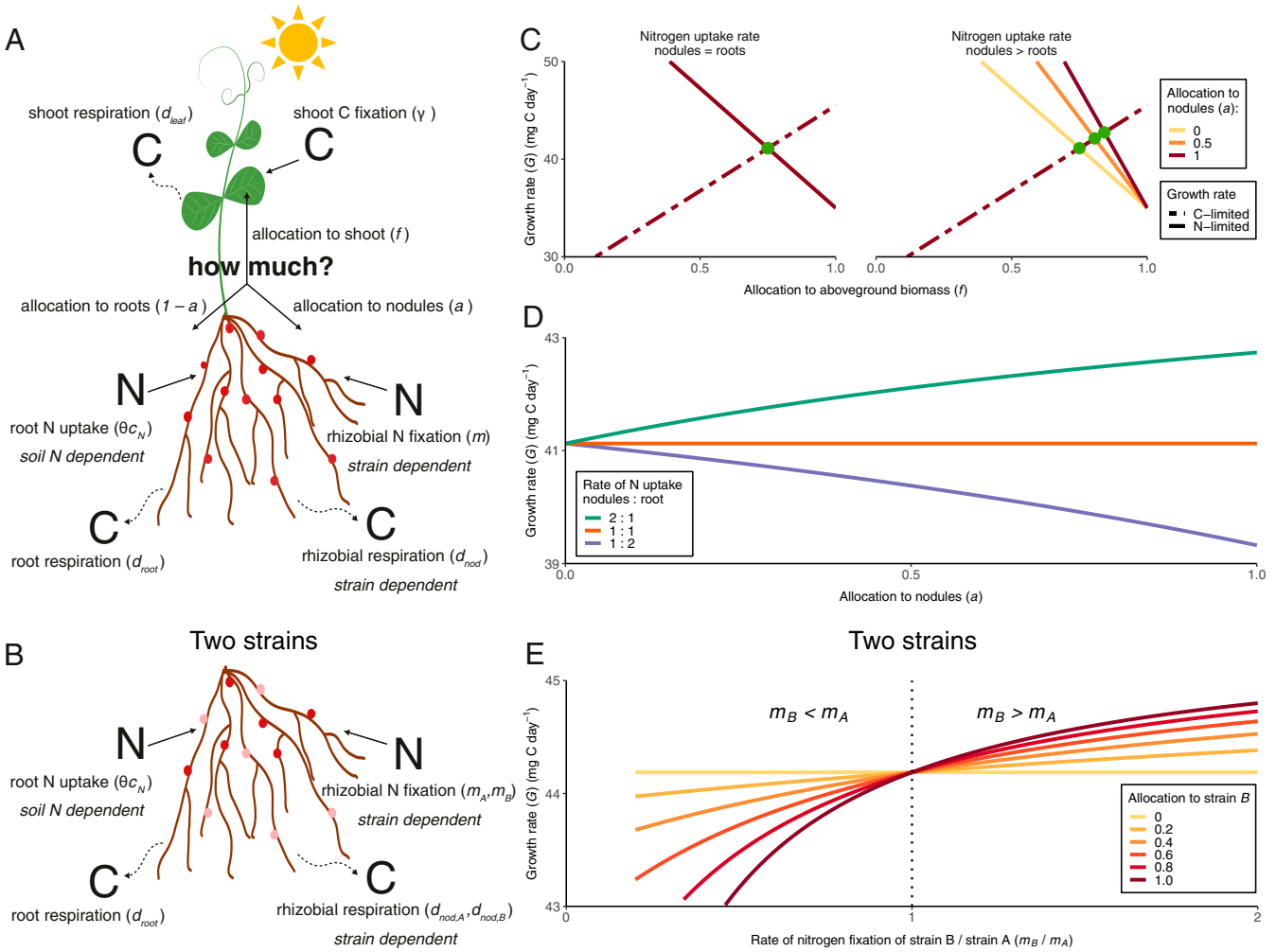


Fig. 1. Schematic representation of the mathematical model for one (*A*) and two (*B*) strains. The model assumes that plant growth is either carbon- or nitrogen-limited and that the plant has perfect knowledge of current carbon and nitrogen availability and demand. The model was used to define how resources should be allocated to shoot, root, and nodules to maximize growth rates. Each rhizobial strain is represented by a single (total) nodule mass, and we do not take into account flowering, seed fill, or time delays between investment in roots or nodules and receiving nitrogen. (*C*) Growth rates are maximized when carbon and nitrogen are equally limiting, and increase with increasing allocation to nodules (*a*) if nodules provide more nitrogen than roots (assuming equal respiration rates). (*D*) Maximum growth rates occur when all resources are allocated to either roots or nodules (except when they are indistinguishable and both sides of Eq. 1 are equal). (*E*) When two strains are both preferred over the root (Eq. 1), growth rates are maximized by either investing in strain A or in strain B, but not both, since intermediate values of allocation to strain B (a_B) are always suboptimal. See *SI Appendix*, Fig. S1 for nonequal respiration rates.

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Thus, we expect that no resources are allocated to a given rhizobial strain beyond a threshold level of soil nitrogen, which is lower for less effective or less efficient strains (*SI Appendix*, Fig. S2 *A* and *B*).

If two rhizobial strains (A and B) meet the condition of Eq. 1, then the plant should invest only in the best available strain. Simultaneous investment in two strains is always suboptimal (Fig. 1*E* and *SI Appendix*, Fig. S1*C*). The plant should invest in strain B rather than strain A when the following occurs:

$$\frac{m_B}{d_{nod,B} + \gamma - d_{leaf}} > \frac{m_A}{d_{nod,A} + \gamma - d_{leaf}}. \quad [2]$$

Thus, both effectiveness (m) and efficiency ($e = d_{nod}/m$) of the two strains determine which strain should be invested in. The relative importance of strain effectiveness and efficiency depends on carbon fixation rates (γ), and efficiency becomes more important when the plant is more carbon-limited (as nodule respiration rates then make up a relatively larger portion of the plant's carbon budget). Crucially, the model predicts that whether or not a plant will invest in a given rhizobial strain is conditional on which other strains are present (Fig. 1*E* and *SI Appendix*, Fig. S1*C* and S2 *C*–*E*). We tested this prediction with strains differing in their ability to fix nitrogen.

Model Test: Nodule Size of an Intermediate-Fixing Strain Depends on Other Strains Present on the Plant. To test whether legumes are able to conditionally sanction rhizobial strains, we inoculated pea plants with different combinations of fixing (Fix^+), intermediate-fixing (Fix^{int}), and nonfixing (Fix^-) derivatives of rhizobial strain Rlv3841, which were otherwise isogenic. Differences in nitrogen fixation were caused by insertions of the Ω Sp^r cassette in either the *nifH* gene (Fix^-) or the region between the *fixX* and *nifA* genes (Fix^{int}) (*SI Appendix*, Fig. S3). Fix^{int} fixes at ~26% of the rate per nodule of Fix^+ (*SI Appendix*, Fig. S4*A*). To discriminate among strains, strains were marked with chromogenic or fluorescent reporters depending on the experiment. In single inoculations, Fix^{int} formed slightly pink nodules of intermediate size (mean 2.37 mm^2): 0.85 mm^2 larger (95% CI [0.07, 1.58]) than Fix^- nodules (mean 1.52 mm^2) and 2.25 mm^2 smaller (95% CI [1.53, 2.97]) than Fix^+ nodules (mean 4.62 mm^2) (Fig. 2 *A*–*C* and *H*).

In three independent experiments, we coinoculated the unmarked Fix^{int} strain with Fix^- (*gusA*-marked), itself (single inoculation), or Fix^+ (*gusA*-marked) and measured the size of the most developed nodules. The nodule size of Fix^{int} was highly dependent on which other strain was present on the plant ($F_{2,36}$ = 60.532, $P < 0.0001$). In the presence of Fix^+, Fix^{int} nodules were less than half the size of nodules on singly inoculated plants, a significant difference of 1.63 mm^2 (95% CI [1.31, 1.95]) (mean 2.99 mm^2 when coinoculated with itself; 1.36 mm^2 with Fix^+) (Fig. 2 *D*–*F* and *I*). In fact, in the presence of Fix^+, Fix^{int} nodules became as small as Fix^- nodules (t_{26} = 0.54658, P = 0.589), consistent with the model prediction that all resources should be allocated to the best available strain. The difference in size between Fix^+ and Fix^{int} was larger when they were coinoculated (Fig. 2*I*), compared with single inoculations (Fig. 2*H*) (significant interaction between strain and inoculation type [singly or coinoculated]; $F_{1,31}$ = 11.9187, P = 0.0016). Results based on randomly selected nodules were in line with those from the most developed nodules (*SI Appendix*, Fig. S5). Fix^{int} typically formed around half of the total nodules on the plant independent of the coinoculated strain ($F_{1,26}$ = 0.1325, P = 0.7188, with Fix^- mean = 44.9%, 95% CI [40.8, 49.0] and with Fix^+ mean = 43.7%, 95% CI [38.9, 48.6]), indicating that it was equally effective at forming nodules.

Finally, we coinoculated all three strains. Fix^{int} nodules (mean 1.74 mm^2) were again similar in size to Fix^- nodules (mean 1.13 mm^2), a nonsignificant difference of 0.61 mm^2 (95% CI [−0.34, 1.56]), but significantly smaller than Fix^+ nodules (mean 6.17 mm^2), with a difference of 4.43 mm^2 (95% CI [3.48, 5.38]) (Fig. 2 *G* and *J*).

Model Test: Carbon Supply to an Intermediate-Fixing Strain Depends on Whether a More Effective Strain Is Also Present. To test whether observed differences in nodule size could be explained by differences in carbon supply to the strains within, we marked the Fix^{int} strain with luminescent biosensors, allowing us to visualize the expression of genes involved in nitrogen fixation and carbon transport. The insertion of *luxCDABE* genes causes rhizobia to luminesce. The *luxCDABE* genes were placed under the control of a specific promoter so that they were coexpressed with other genes at key stages of nodule development. The P*nifH* biosensor luminesces when nitrogenase genes are expressed during nodule development. The C4-dicarboxylates and sucrose biosensors are indicators of carbon supply to the strains within a nodule, as they luminesce when the *dctA* transporter (transports C4-dicarboxylates) and the sucrose transporter are transcribed (17). Nonfixing nodules in single inoculations have been shown to have lower expression levels of these transporter genes as their transcription responds to the presence of the relevant substrates which are provided by the plant (17).

In two independent experiments, we calculated the percentage of Fix^{int} nodules which were expressing luminescence over a background level of 40 cps, to determine the percentage of nodules with active carbon transport. We found that in the early stages of nodule development (12 dpi), when only a few nodules had started to express nitrogenase genes (Fig. 3*A*), carbon was supplied to almost all Fix^{int} nodules, regardless of whether the plant was coinoculated with Fix^- or Fix^+ (nonsignificant [ns] difference with odds ratio [OR] 2.27 for C4-dicarboxylates, 95% CI [0.09, 56.77], and OR 0.45 for sucrose, 95% CI [0.03, 7.57]) (Fig. 3 *B* and *C*). At a slightly later developmental stage (20 dpi), most nodules were expressing nitrogenase genes (71.8%, 95% CI [59.3, 81.6]) (Fig. 3*A*), which allows the plants to discriminate between the fixation abilities of strains. At this stage, a smaller percentage of Fix^{int} nodules showed active carbon transport when coinoculated with Fix^+, compared with with Fix^- (note that we would not expect none of the nodules to receive carbon, since pea plants nodulate continuously and therefore some nodules will be at an earlier developmental stage). The difference was significant in the case of sucrose (OR 2.81, 95% CI [1.22, 6.43]) but not quite significant in the case of C4-dicarboxylates (OR 1.99, 95% CI [0.81, 4.93]) (Fig. 3 *B* and *C*). Thus, differences in carbon supply to nodules (20 dpi; Fig. 3 *B* and *C*) preceded differences in nodule size (28 dpi; Fig. 2 *H*–*J*). Taken together, these results indicate that pea plants conditionally sanction intermediate-fixing strains based on which other strains are present.

Conditional Resource Allocation Affects Nitrogen Fixation Rate and Rhizobial Viability in Intermediate-Fixing Strains. Finally, we looked at how the conditional supply of carbon to intermediate-fixing strains affected nitrogen fixation rates (using acetylene reductions) and rhizobial fitness (using plate counts). In two independent experiments, we coinoculated a mini-Tn7 mCherry-marked Fix^{int} strain with either a mini-Tn7 green fluorescent protein (GFP)-marked Fix^-, Fix^{int}, or Fix^+ strain to identify strains without damaging the nodules.

To assess nitrogen fixation rates, we performed acetylene reductions at 28 dpi after removing all nodules containing GFP-marked strains, leaving only nodules containing the mCherry-marked Fix^{int} strain. The per nodule rate of nitrogen fixation of Fix^{int} was dependent on the identity of the coinoculated strain ($F_{2,20}$ = 7.2161, P = 0.0044). Fix^{int} fixed significantly less nitrogen when coinoculated with Fix^+, compared with itself (a difference of 0.0018, 95% CI [0.0005, 0.0032] μmol ethylene $nodule^{-1} \cdot hour^{-1}$)

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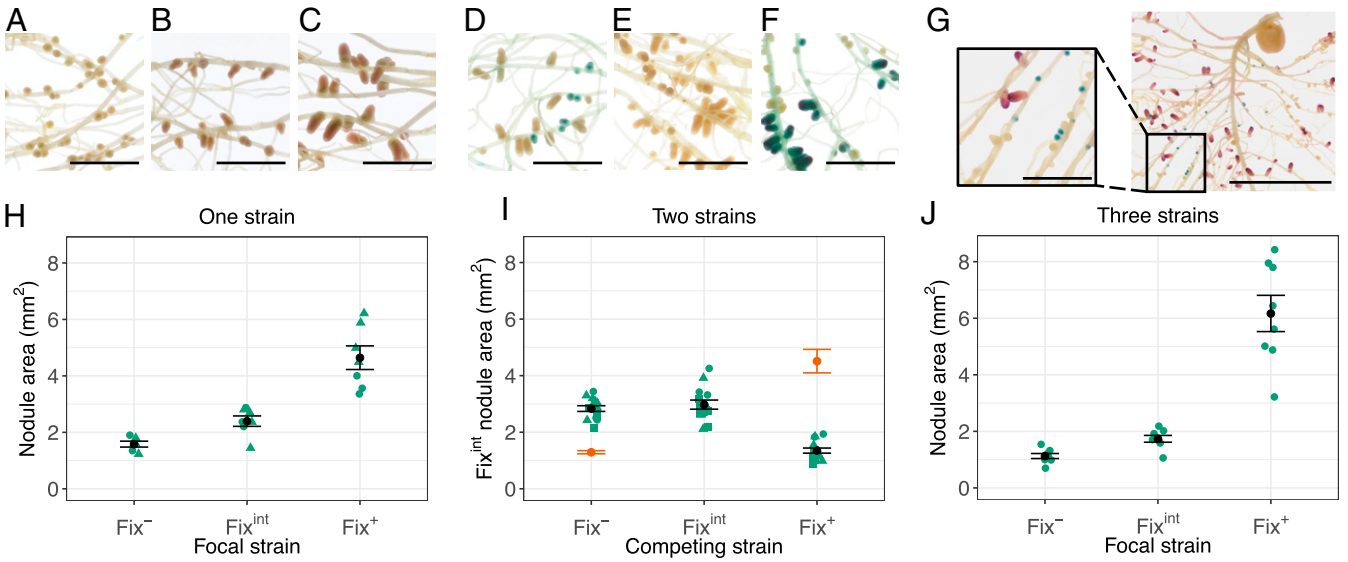


Fig. 2. Nodule size of an intermediate-fixing strain is conditional on which other strains are present on the plant. Pea roots were inoculated with different combinations of nonfixing (Fix$^-$), intermediate-fixing (Fix^{int}), and fixing (Fix$^+$) strains. In single inoculations, Fix$^-$ nodules were small and white (*A*), Fix^{int} nodules were slightly pink and intermediate in size (*B*) and Fix$^+$ nodules were the darkest and largest (*C*). In coinoculations, the nodules of the Fix^{int} strain (unmarked) were large when coinoculated with Fix$^-$ (*gusA*-marked, blue) (*D*), or itself (*E*), but small when with Fix$^+$ (*gusA*-marked, blue) (*F*). When all three strains were coinoculated (*G*) (Fix$^-$ *celB*-marked, blue; and Fix$^+$ *gusA*-marked, magenta), Fix^{int} nodules were as small as Fix$^-$ nodules, while the Fix$^+$ nodules were more than twice as large. Nodule areas in single inoculations (*H*), coinoculations (*I*), and triple inoculations (*J*) are also shown. Points represent an average of 10 nodules per strain and per independent replicate plant. Symbols indicate data from independent experiments. Black points indicate the overall mean with one SE; orange points indicate this for competitor nodules on the same plants. Plants were grown for 27 to 28 d. (Scale bars, 10 mm (*A–F, inset G*) and 30 mm (*G*).) n = 6 to 7 (*H*), n = 14 (*I*), and n = 8 (*J*).

or with Fix$^-$ (a difference of 0.0025, 95% CI [0.0012, 0.0039] μmol ethylene nodule^{-1} · hour^{-1}) (Fig. 3*D*). Thus, the observed reduction in carbon allocation to Fix^{int} nodules on plants coinoculated with Fix$^+$ (Fig. 3 *B* and *C*) also resulted in a reduction in per nodule nitrogen fixation rates.

The number of viable rhizobia per Fix^{int} nodule was dependent on the coinoculated strain and the age of the plant at harvest (interaction $F_{2,26} = 4.612$, $P = 0.019$). Despite observable differences in nodule size and carbon allocation by 28 dpi (Figs. 2 and 3 *B* and *C*), we did not observe differences in the number of viable rhizobia per nodule at this developmental stage. Fix^{int} had similar numbers of viable rhizobia per nodule when coinoculated with Fix$^+$, compared with itself (ns difference of 6.357×10^5, 95% CI [-6.259×10^6, 7.530×10^6]) or with Fix$^-$ (ns difference of 5.071×10^5, 95% CI [-6.387×10^6, 7.401×10^6]) (Fig. 3*E*). However, at 56 dpi, Fix^{int} had significantly fewer viable rhizobia per nodule when coinoculated with Fix$^+$, compared with itself (a difference of 1.021×10^7, 95% CI [0.301×10^7, 1.741×10^7]) or with Fix$^-$ (a difference of 1.318×10^7, 95% CI [0.629×10^7, 2.008×10^7]) (Fig. 3*F*).

These results were corroborated by light microscopy imaging. We found that Fix^{int} nodules from plants coinoculated with Fix$^+$ or Fix$^-$ differed in key ways: When coinoculated with Fix$^+$, Fix^{int} nodules were seen to senesce prematurely, had no active meristem, and had a smaller infected zone III where nitrogen fixation takes place, although cells still appeared healthier than Fix$^-$ cells (*SI Appendix*, Fig. S6).

Discussion

Our model predicted that optimal allocation to multiple rhizobial strains requires conditional investment decisions, so that plants should only allocate resources to the best available strain. Model predictions were supported by a series of experiments, in which investment in an otherwise isogenic intermediate-fixing strain was conditional on whether a more effective strain was also available. Initially, carbon transport to intermediate-fixing rhizobia was independent of the coinoculated strain. This makes sense, as even nodules containing a superior strain provide little or no nitrogen at the start of nodule development, so discrimination must be dependent on the stage of nodule development. However, as nodules developed, carbon transport to an intermediate-fixing strain was reduced and nodules containing this strain were smaller, with reduced nitrogen fixation—but only when a better (more effective) alternative was available to the plant. At even later developmental stages, this reduction in resource allocation resulted in lower rhizobial fitness.

Our finding that resource allocation to intermediate-fixing nodules is dependent on which other strain is present implies that there must be a systemic mechanism to compare the effectiveness of different nodules and adjust resource allocation accordingly. Previous observations of sanctions against nonfixing strains (9–11) could be explained by a model in which resource allocation to nodules is simply proportional to their effectiveness. Such a mechanism could operate at the level of individual nodules, without any need for integration across the plant, or even within nodules containing both effective and ineffective strains (18, 19). However, we now show that the plant response to an intermediate-fixing strain cannot be explained exclusively at the cell or nodule level (for example, through an immune response), as could be argued for a completely nonfixing strain (20), as the response was conditional on coinoculated strains.

The simplest way in which plants could evaluate nodules (or possibly cells within nodules) is by comparing concentrations of a cue in the nodule with systemic concentrations of the same cue. The nodule concentration of the cue is determined by local rates of nitrogen fixation, whereas the systemic concentration is determined by the overall plant nitrogen status, and the difference determines nodule development. This mechanism is consistent with the findings that more effective nodules are larger in single inoculations and that addition of soil nitrogen reduces resource

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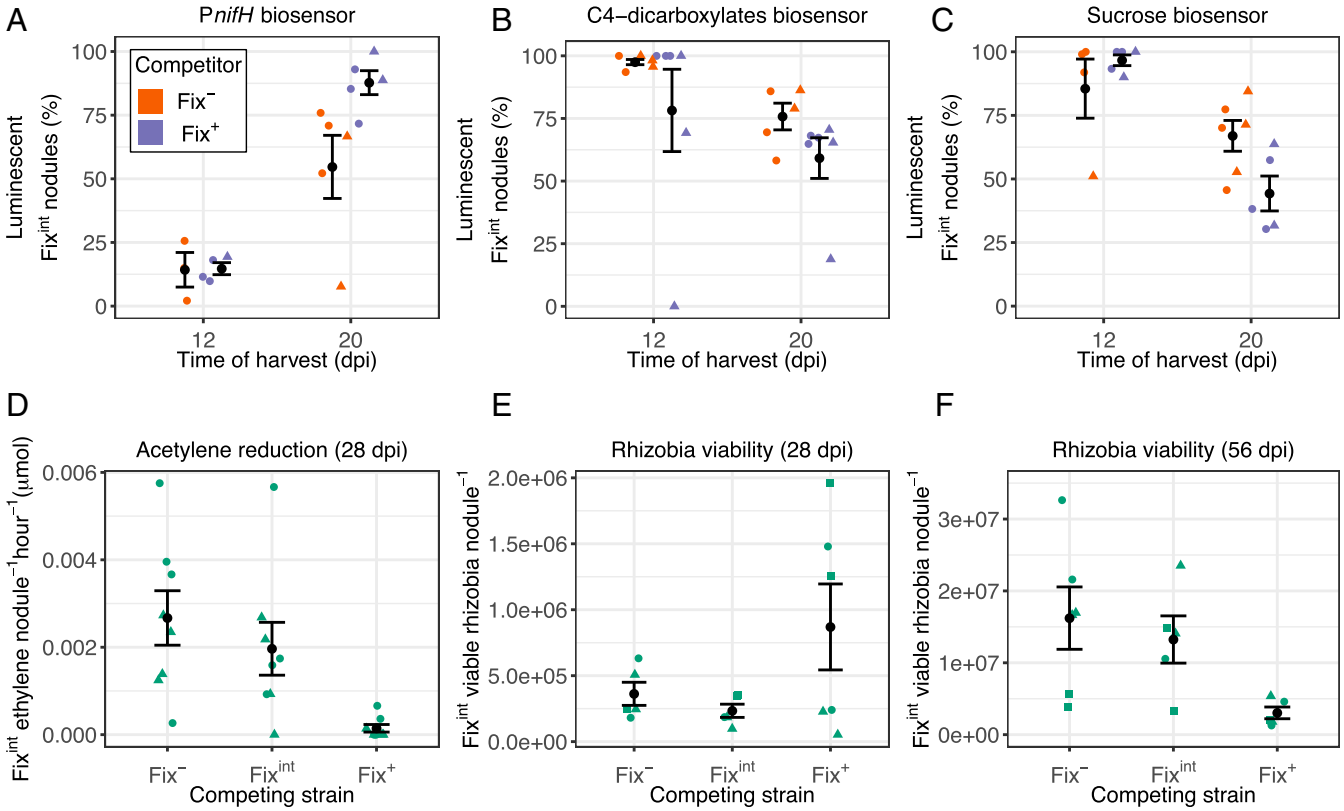


Fig. 3. Sucrose supply, acetylene reduction rates, and rhizobia viability of an intermediate-fixing strain are conditional on which other strains are present on the plant. Pea roots were inoculated with Fix^{int}, in combination with Fix^{-}, itself, or Fix^{+}. At 12 dpi, the percentage of luminescent Fix^{int} nodules did not depend on the coinoculated strain for any of the luminescent biosensors: P*nifH* (*A*), C4-dicarboxylates (*B*), and sucrose (*C*). At 20 dpi, when most nodules were fixing nitrogen (*A*), Fix^{int} marked with the sucrose biosensor had significantly more luminescent nodules when coinoculated with Fix^{-} compared with Fix^{+} (*C*). (*D*) At 28 dpi, Fix^{int} fixed more nitrogen per nodule when coinoculated with Fix^{-} or itself, compared with Fix^{+}. The reduction in viable rhizobia when Fix^{int} was coinoculated with Fix^{+} was not apparent at 28 dpi (*E*) but very clear at 56 dpi (*F*) (note the scale difference). Points represent independent replicate plants, an average of three technical replicates (*D*), or five technical replicates of 10 nodules combined (*E* and *F*). Symbols indicate data from independent experiments. Black points represent the overall mean with one SE. $n = 3$ to 5 (*A*), $n = 4$ to 6 (*C*), $n = 8$ (*D*), and $n = 5$ to 6 (*B*, *E*, and *F*).

allocation to both effective and less effective strains (16, 21). Such systemic control is also consistent with recent findings in split-root systems that whole-plant changes in nitrogen status rapidly result in changes to carbon allocation and nodule metabolism in established nodules (22).

Our model also predicted conditional sanctioning based on efficiency. Strains may differ in how much carbon they use per unit of nitrogen fixed; for example, if carbon is stored. Recent findings by others do not provide evidence for conditional sanctioning based on efficiency (23) but given our model prediction that efficiency becomes more important when the plant is carbon-limited, it would be worthwhile to conduct experiments in which the plant is carbon- rather than nitrogen-limited. Other additional experiments could use different inoculation ratios of strains differing in effectiveness (or efficiency), because when the best available strain is rare, the plant may invest in suboptimal strains. Longer term experiments assessing the consequences of conditional sanctioning for rhizobial fitness would also be worthwhile, as conditional sanctioning may help explain the recently described evolution toward more cooperative strains in an experimental setting (24). Additionally, it would be interesting to test our predictions in other symbioses. Hosts may be more likely to conditionally sanction when they are less dependent on the symbiosis (25), when symbionts are compartmentalized (26), or when there are fewer alternative partners for the host to choose from (27).

Conditional sanctioning allows legumes to discriminate between rhizobia, giving them remarkable control over their symbiotic partners, which is important in systems where poor fixers are common. It has been argued that the regulation of resource allocation to nodules is derived from preexisting mechanisms regulating root growth (28). If so, our findings may apply widely, and elucidating the mechanisms may help to understand root system architecture more generally and to select for legumes which are better at discriminating among strains.

Materials and Methods

Model Formulation. The theoretical model is based on a previous model considering plant growth and allocation in the absence of rhizobia (29). Key assumptions of the model are the following: 1) plant growth is either carbon- or nitrogen-limited; and 2) the plant has perfect knowledge of current carbon and nitrogen uptake rates and demands but has no information about future conditions.

The carbon-limited growth rate (G_C) at a given point in time depends on photosynthesis—the product of the carbon uptake rate per gram shoot (γ) and the current biomass of aboveground tissue (M_{leaf})—minus the carbon demands of existing shoot (d_{leaf}), root (d_{root}), and nodules (d_{nod}) (Eq. 3.1.a). Thus, carbon demands of existing structures are met before resources can be allocated to new growth and the model does not include senescence and redistribution of the resulting resources. The nitrogen-limited growth rate (G_N) is given by the sum of root nitrogen uptake and nitrogen fixation by nodules. Root nitrogen uptake depends on current root biomass (M_{root}), soil nitrogen concentration (c_N), and a coefficient giving the uptake per gram root given the soil nitrogen concentration (soil exploration rate) (θ), while nitrogen fixation is given by nodule mass (M_{nod}) and the nitrogen fixation rates (m) of different strains (two strains shown, but the model could be extended to include any number of strains). The terms in the nitrogen-

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limited growth rate are multiplied by the tissue C:N ratio (ρ) so that both growth rates are expressed as mg carbon · day^{-1} (Eq. 3.1.b):

$$G_C(t) = \gamma M_{leaf}(t) - d_{leaf}M_{leaf}(t) - d_{root}M_{root}(t) - d_{nod,A}M_{nod,A}(t) - d_{nod,B}M_{nod,B}(t) \quad [3.1.a]$$

and

$$G_N(t) = \rho\theta c_N(t)M_{root}(t) + \rho m_A M_{nod,A}(t) + \rho m_B M_{nod,B}(t). \quad [3.1.b]$$

The actual growth rate achieved is the minimum of the carbon-limited and nitrogen-limited growth rates (Eq. 3.2.a):

$$G(t) = min\,(G_C(t),\ G_N(t)). \quad [3.2.a]$$

Growth is then distributed over shoot, root, and nodules of different types (Eq. 3.3). A fraction (*f*) of the total growth is allocated to aboveground biomass (Eq. 3.3.a), and the remainder (1 − *f*) to belowground biomass. The growth is split between roots and nodules by allocating a fraction (*a*) to nodules and the remainder to roots (1 − a_A − a_B) (Eq. 3.3.b–d):

$$\frac{dM_{leaf}}{dt} = f(t)G(t), \quad [3.3.a]$$

$$\frac{dM_{root}}{dt} = (1 - f(t))\,(1 - a_A(t) - a_B(t))\,G(t), \quad [3.3.b]$$

$$\frac{dM_{nod,A}}{dt} = (1 - f(t))\,a_A(t)\,G(t), \quad [3.3.c]$$

and

$$\frac{dM_{nod,B}}{dt} = (1 - f(t))\,a_B(t)\,G(t). \quad [3.3.d]$$

Model Implementation. The model was implemented in R version 3.3.2 (30) to check analytical solutions and to generate graphs, with parameter values as presented in *SI Appendix*, Table S1. Results are independent of these specific parameter values since all results are based on analytical solutions for optimal values of allocation parameters *f* (allocation to aboveground biomass) and *a* (allocation to nodules).

Model Analyses. All results are based on analytical solutions, which were checked numerically. The optimal value of allocation to aboveground biomass (*f*) was found by defining the growth rate at time $t + \Delta t$ and assuming that maximum growth rate is achieved when carbon and nitrogen are equally limiting at all times, following the approach used in Guilbaud et al., 2014 (29) (*SI Appendix*). Once the optimal value of allocation to aboveground biomass (*f*) was found analytically, it was used in all subsequent analyses, and to determine initial values of above- and belowground biomass in the model implementation (*SI Appendix*). The optimal value of allocation to nodules (*a*) was found by again considering growth rates at time $t + \Delta t$ and asking when investing in nodules leads to higher growth rates than not investing in nodules ($G(t + \Delta)_{a=1} > G(t + \Delta t)_{a=0}$, *SI Appendix*).

Bacterial Strains and Culture Conditions. Rhizobial strains used in this study are all derivatives of Rlv3841 (31) (*SI Appendix*, Table S2). Rhizobial strains (*SI Appendix*, Table S2) were maintained on tryptone-yeast (TY) agar (32) and incubated at 28 °C, and *Escherichia coli* strains (*SI Appendix*, Table S3) were maintained on Luria-Bertani (LB) agar (33) and incubated at 37 °C, both with the appropriate concentrations of antibiotics (*SI Appendix*, Tables S2 and S3) and stored at 7 °C. Plasmids in ST18 backgrounds (pOPS1526 and pOPS1531) were supplemented with 50 μg mL^{-1} 5-aminolevulinic acid (ALA) as ST18 cells are ALA auxotrophs (34). For long-term storage, strains were kept at −80 °C in TY or LB medium as appropriate with 15 to 20% glycerol. Rhizobial inocula consisted of a suspension of rhizobia grown on TY agar (32) diluted to a concentration of 1 × 10^2 cells mL^{-1} (most experiments) or 1 × 10^4 cells mL^{-1} (fluorescent strains). The details of cloning methods to make the intermediate-fixing strain and the strains marked with *lux* or fluorescent reporters are described in *SI Appendix*.

Plant Growth. Pea (*Pisum sativum* L. cv. Avola) seeds were surface sterilized (30 s in 95% ethanol followed by 5 min in 2% NaClO), rinsed, and left to germinate on 0.8% agar plates at room temperature in the dark. Seedlings were planted after 5 d by transferring them to sterilized 1-L (viability experiment) or 500 mL (all other experiments) Azlon beakers containing a mixture of silver sand and fine vermiculite (1:1 vol/vol), 150 mL or 75 mL (for 1-L or 500 mL beakers respectively) sterilized nitrogen-free nutrient solution [as in Poole et al. (35) but 2.67 times more concentrated], and 1 mL of rhizobial inoculum. Beakers were covered with cling film to prevent contamination, which was slit after a few days to allow seedlings to grow. Plants were grown in the growth room (21 °C, 16 h photoperiod) for 12 to 56 d and watered as necessary from 7 d onwards.

Harvest. At harvest, plants were gently removed from the pots and shaken to remove any clinging substrate.

For GusA and CelB staining (36, 37), roots were gently washed with water, and submerged in 40 mL phosphate buffer (7 g L^{-1} NaH_2PO_4, 7.2 g L^{-1} Na_2HPO_4, 1 mM ethylenediaminetetraacetic acid [EDTA] [pH 8], 1% Sarkosyl, 1 mL L^{-1} Triton) in Falcon tubes covered with aluminum foil. For GusA staining, Magenta-glcA (5-bromo-6-chloro-3-indolyl-β-D-glucuronide) or X-glcA (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) (blue) was added to a final concentration of 0.2 mg mL^{-1} and roots were incubated overnight at 28 °C. If additional CelB staining was necessary, roots were then transferred to fresh preheated phosphate buffer and incubated for ± 2 h at 70 °C to destroy endogenous β-galactosidase activity, after which X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) (blue) was added to a final concentration of 0.25 mg mL^{-1} and roots were incubated overnight at 37 °C. Roots were then carefully laid out on 120 × 120 mm^2 plates so that all nodules were visible and photographed for further data collection using a Nikon D300 or D700 camera with no filter, no flash, and exposure time as needed.

For detecting light emission from strains containing luminescent biosensors, roots were gently washed and carefully laid out on 120 × 120 mm^2 plates and imaged with a NightOwl II camera (Berthold Technologies) at an exposure time of 60 s using inbuilt IndiGO software v2.0.5.0. Nodules emitting over 40 cps of luminescence (to avoid background noise) were detected and counted automatically using the IndiGO software with manual curation to ensure the luminescent areas aligned with the root system.

Afterward (or directly if using strains with no luminescent biosensors), to discriminate between the fluorescently marked nodules, roots were placed on a blue-light transilluminator (VWR) and photographed using a Nikon D700 camera (55 mm Micro-Nikkor f/2.8 lens, orange filter [Hoya Orange (G)], f/22 aperture, and 10 s exposure) for further data collection.

For acetylene reductions, plants were transferred to 250 mL Duran bottles with airtight neoprene lids. Bottles contained some wet tissue paper to prevent drying of the samples. Acetylene was added with a syringe through the lid to a final concentration of 2%. We used this low concentration to minimize the risk of errors due to the effect of acetylene on nitrogenase activity (38). After one hour incubation at room temperature, three samples were taken from the jar and analyzed for acetylene and ethylene by gas chromatography (Clarus 480, PerkinElmer). For acetylene reductions of plants inoculated with two strains, a transilluminator was used to identify the fluorescent markers as above, and all GFP nodules were removed. Plants were kept moist during this process, and the order of plants to have their nodules removed was randomized. Plants then underwent acetylene reduction.

To measure rhizobial fitness, 10 GFP and 10 mCherry nodules were identified from transilluminator images taken as above, surface sterilized (60 s in 2% NaClO), washed five times in sterile H_2O, and crushed in 1 mL sterile bacteroid isolation buffer (8.2 mM K_2HPO_4, 1.98 mM KH_2PO_4, 300 mM sucrose, and 2 mM $MgCl_2$). Serial dilutions were drop plated on TY with streptomycin (500 μg · ml^{-1}) and nystatin (50 μg · ml^{-1}), and colonies were counted after 2 d growth.

Data Collection Nodule Counts and Sizes. From the photographs taken of the root systems, the number of nodules formed by each strain was counted. Strains were discriminated (if necessary) based on GusA or CelB staining or GFP or mCherry expression. Nodule size per plant and per strain was estimated by measuring the area of the 10 most developed (largest) nodules, so that variation in size due to developmental stage was accounted for (10 randomly selected nodules gave similar results, *SI Appendix*, Fig. S5). Using the software ImageJ v1.49v and 1.53a (39), areas of irregular shapes could be accurately measured.

Experimental Designs. To test how plants allocate resources among strains differing in how much nitrogen they provide, the Fix^{int} strain (OPS1474) was co-inoculated with the Fix$^-$ strain (OPS0365), itself, or the Fix$^+$ strain (Rlv3841 *gusA*) by inoculating plants with 1:1 mixtures of the strains at a total density of 1 × 10^2 cells per pot [a low density to minimize the occurrence of mixed infection nodules (11)]. Three independent experiments were conducted, consisting of five replicate plants (two experiments) or four replicate plants (one experiment) per treatment, plus two water controls grown in a fully randomized design. Resource allocation among three strains was tested by inoculating plants with a 1:1:1 mixture of the

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Fix^+ strain (Rlv3841 *gusA*), the Fix^{int} strain (OPS1474), and a Fix^- strain (OPS0366) at a total density of 1×10^2 cells per pot. Eight plants and four water controls were grown in a fully randomized design, along with single inoculations of each strain (four replicates). The single inoculations in this experiment were also used to measure nitrogen fixation rates of singly inoculated strains. In an additional experiment, plants received single inoculations of the Fix^- (OPS0365), Fix^{int} (OPS1474), or Fix^+ (Rlv3841 *gusA*) strains (three replicates).

To test whether conditional plant resource allocation could be observed in carbon supply to the nodule, a GFP and luminescent biosensor double-marked Fix^{int} strain (P*nifH* OPS2314, C4-dicarboxylates OPS2313, or sucrose OPS2278) was coinoculated with a mCherry and empty plasmid (pJP2, the same plasmid backbone as the biosensors) marked Fix^- strain (OPS2273), or Fix^+ strain (OPS2272) in all possible combinations by inoculating plants with 1:1 mixtures of the strains at a total density of 1×10^4 cells per pot. Two independent experiments were conducted, each consisting of three replicate plants per treatment per time point, plus two water controls per time point grown in a fully randomized design.

To measure nitrogen fixation rates of the Fix^{int} strain, depending on which strain it was coinoculated with, a mCherry-marked Fix^{int} strain (OPS2269) was coinoculated with a GFP-marked Fix^- strain (OPS2270), Fix^{int} strain (OPS2268), or Fix^+ strain (OPS1339) by inoculating plants with 1:1 mixtures of the strains at a total density of 1×10^4 cells per pot. Two independent experiments were conducted, each consisting of four replicate plants per treatment, plus four water controls grown in a fully randomized design.

To measure rhizobial fitness of the Fix^{int} strain, depending on which strain it was coinoculated with, a Fix^{int} strain was coinoculated with a Fix^-, Fix^{int}, or Fix^+ strain. The Fix^{int} strain was GFP-marked (OPS2268) and coinoculated with mCherry-marked Fix^-, Fix^{int} or Fix^+ strains (OPS2271, OPS2269, or OPS1341, respectively), or mCherry-marked (OPS2269) and coinoculated with GFP-marked Fix^-, Fix^{int}, or Fix^+ strains (OPS2270, OPS2268, or OPS1339, respectively). Plants were inoculated with 1:1 mixtures of the strains at a total density of 1×10^4 cells per pot. Three independent experiments were conducted, each consisting of two replicate plants per coinoculated strain (GFP-marked Fix^{int} strain with mCherry-marked coinoculated strain and vice versa) per time point, plus two water controls grown in a fully randomized design.

Statistical Analyses. Analyses were conducted using R version 3.6.3 (40). For mixed-effects models, lmer from the package lme4 version 1.1-23 (41) was used to fit models and the package lmerTest version 3.1-2 (42) was used to provide *P* values.

For all analyses of nodule size, a single data point per plant and per strain was obtained by averaging the nodule area measurements of the 10 most developed (largest) nodules per strain. Experiments were often repeated and results from multiple experiments were combined for analysis (they were always very similar). Nodule sizes of strains in single inoculations were analyzed as a function of strain identity in a linear mixed-effects model with experiment as the random effect. Nodule sizes of the intermediate-fixing strain in double inoculations were analyzed in a linear mixed-effects model with the identity of the coinoculated strain as the fixed effect and experiment as the random effect. To compare the size of nonfixing nodules with nodules of the intermediate-fixing strain in the presence of the fixing strain, a two-sample *t* test was performed. To test whether the difference in nodule size between the intermediate-fixing strain and the fixing strain depended on whether they were coinoculated or not, a linear mixed-effects model was fitted, where the fixed effects are: strain (Fix^{int} or Fix^+), inoculation type (single or coinoculation) and their interaction, and block as the random effect (each coinoculation experiment was conducted at the same time as a single inoculation experiment). The percentage of nodules formed by the intermediate-fixing strain was analyzed as a function of coinoculated strain (Fix^- or Fix^+) with a generalized linear model using a quasibinomial distribution. The triple inoculation experiment was analyzed as a mixed-effects model, including plant as a random effect, to account for the fact that on each plant nodule sizes of all three strains were measured.

To analyze differences in carbon transport to rhizobia, we calculated the percentage of intermediate-fixing nodules emitting light (thus expressing *luxCDABE*) for each biosensor (P*nifH*, C4-dicarboxylates, or sucrose) and time point (12 and 20 dpi). The percentage of nodules emitting light was analyzed in separate generalized linear models for each biosensor, with a quasibinomial distribution where the fixed effects are the identity of the coinoculated strain, time point, and their interaction.

For rates of acetylene reduction, a single data point per plant was obtained by averaging three technical replicates. For single inoculations, rates were analyzed as a function of strain using a linear model. For coinoculations, rates were analyzed using a linear mixed-effects model, where the identity of the coinoculated strain is the fixed effect and experiment is the random effect.

The fitness of rhizobia was expressed as the number of viable rhizobia per nodule by averaging five technical replicates of drop counts from 10 nodules combined and analyzed in a linear model as a function of coinoculated strain, time point (28 and 56 dpi), and the interaction.

Data Availability. The data, images, and R code that support the findings of this study are available in the Oxford University Research Archive repository (https://doi.org/10.5287/bodleian:6qmxaR7db) (43).

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Supplementary Information for

Conditional sanctioning in a legume-*Rhizobium* mutualism

Authors: Annet Westhoek[1], Laura J. Clark[1], Michael Culbert, Neil Dalchau, Megan Griffiths, Beatriz Jorrin, Ramakrishnan Karunakaran, Raphael Ledermann, Andrzej Tkacz, Isabel Webb, Euan K. James, Philip S. Poole[*], Lindsay A. Turnbull[*].
[1]These authors contributed equally.

**Corresponding Authors:* Lindsay A. Turnbull (+44(0)1865275145) and Philip S. Poole (+44(0)1865275023). Department of Plant Sciences, University of Oxford, Oxford, OX1 3RB, United Kingdom

Email: lindsay.turnbull@plants.ox.ac.uk philip.poole@plants.ox.ac.uk

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Supplementary Information Text

Derivation of optimal allocation to aboveground biomass (*f*). The derivation of the optimal allocation of growth to the shoot is based on the assumption that optimal growth is achieved when carbon and nitrogen are equally limiting, following the approach of Guilbaud *et al*., 2014 (1):

$$G_C(t) = G_N(t) \quad \textbf{[S1.1a]}$$

$$G_C(t+\Delta t) = G_N(t+\Delta t) \quad \textbf{[S1.1b]}$$

where growth at time $t + \Delta t$ (in the case of a single rhizobial strain) is defined as:

$$G_C(t+\Delta t) = \gamma M_{leaf}(t+\Delta t) - d_{leaf} M_{leaf}(t+\Delta t) - d_{root} M_{root}(t+\Delta t) - d_{nod} M_{nod}(t+\Delta t) \quad \textbf{[S1.2a]}$$

$$G_N(t+\Delta t) = \rho\theta M_{root}(t+\Delta t) c_N + \rho m M_{nod}(t+\Delta t) \quad \textbf{[S1.2b]}$$

in which

$$M_{leaf}(t+\Delta t) = M_{leaf}(t) + f(t)G(t)\Delta t \quad \textbf{[S1.3a]}$$

$$M_{root}(t+\Delta t) = M_{root}(t) + (1-f(t))(1-a(t))G(t)\Delta t \quad \textbf{[S1.3b]}$$

$$M_{nod}(t+\Delta t) = M_{nod}(t) + (1-f(t))a(t)G(t)\Delta t \quad \textbf{[S1.3c]}$$

Substituting Eq. **S1.3** into Eq. **S1.2** into Eq. **S1.1**, cancelling out $G_C(t) = G_N(t)$ and dividing by $G(t)\Delta t$ leads to:

$$\gamma f(t) - d_{leaf} f(t) - d_{root}\left(1 - a(t) - f(t) - a(t)f(t)\right) - a(t) d_{nod} + a(t) f(t) d_{nod} = \rho\theta c_N \left(1 - f(t) - a(t) + a(t) f(t)\right) + \rho m a(t) - \rho m f(t) a(t) \quad \textbf{[S1.4]}$$

Rearranging results in an equation for optimal allocation to shoot growth:

$$f = \frac{d_{root} + \rho\theta c_N + a\left(d_{nod} - d_{root} + \rho\left(m - \theta c_N\right)\right)}{\gamma - d_{leaf} + d_{root} + \rho\theta c_N + a\left(d_{nod} - d_{root} + \rho\left(m - \theta c_N\right)\right)} \quad \textbf{[S1.5]}$$

Following the same approach for the case of two rhizobial strains leads to:

$$f = \frac{d_{root} + \rho\theta c_N + a_A(d_{nod,A} - d_{root} + \rho m_A - \rho\theta c_N) + a_B(d_{nod,B} - d_{root} + \rho m_B - \rho\theta c_N)}{\gamma - d_{leaf} + a_A(d_{nod,A} - d_{root} + \rho m_A - \rho\theta c_N) + a_B(d_{nod,B} - d_{root} + \rho m_B - \rho\theta c_N)} \quad \textbf{[S1.6]}$$

Derivation of initial conditions. Derivation of the initial shoot and root mass follows the approach of Guilbaud *et al.,* 2014 and is based on the assumptions that (*i*) carbon and nitrogen are equally limiting growth at time $t = 0$, (*ii*) initially there are no nodules, and (*iii*) the combined initial root and shoot mass must equal the seed mass. Therefore, at time $t = 0$:

$$G_C = G_N \quad \textbf{[S2.1]}$$

$$M_{seed} = M^0_{leaf} + M^0_{root} \quad \textbf{[S2.2]}$$

This means that:

$$\gamma M^0_{leaf} - d_{leaf} M^0_{leaf} - d_{root} M^0_{root} = \rho\theta M^0_{root} c^0_N \quad \textbf{[S2.3]}$$

Rearranging gives:

$$M^0_{leaf} = \frac{M^0_{root}\left(\rho\theta c_N + d_{root}\right)}{\gamma - d_{leaf}} \quad \textbf{[S2.4a]}$$

$$M^0_{root} = \frac{M^0_{leaf}\left(\gamma - d_{leaf}\right)}{\rho\theta c_N + d_{root}} \quad \textbf{[S2.4b]}$$

Substituting $M^0_{root} = M_{seed} - M^0_{leaf}$ into Eq. **S2.4a** and $M^0_{leaf} = M_{seed} - M^0_{root}$ into Eq. **S2.4b** and rearranging leads to the initial conditions:

$$M^0_{leaf} = \frac{\rho\theta c_{N_0} + d_{root}}{\rho\theta c_{N_0} + \gamma + d_{root} - d_{leaf}} M_{seed} \quad \textbf{[S2.5a]}$$

$$M^0_{root} = \frac{\gamma - d_{leaf}}{\rho\theta c_{N_0} + \gamma + d_{root} - d_{leaf}} M_{seed} \quad \textbf{[S2.5b]}$$

Derivation of optimal allocation to nodules (*a*) for a single rhizobial strain. Optimal allocation to nodules (the value of parameter *a*) was found by considering the carbon-limited (G_C) and nitrogen-limited (G_N) growth rates at time $t + \Delta t$. Growth at time $t + \Delta t$ (in the case of a single rhizobial strain) is defined as:

$$G_C\left(t+\Delta t\right)=\gamma M_{leaf}\left(t+\Delta t\right)-d_{leaf}M_{leaf}\left(t+\Delta t\right)-d_{root}M_{root}\left(t+\Delta t\right)-d_{nod}M_{nod}\left(t+\Delta t\right) \quad \textbf{[S3.1a]}$$

$$G_N\left(t+\Delta t\right)=\rho\theta M_{root}\left(t+\Delta t\right)c_N+\rho m M_{nod}\left(t+\Delta t\right) \quad \textbf{[S3.1b]}$$

in which

$$M_{leaf}\left(t+\Delta t\right)=M_{leaf}\left(t\right)+f\left(t\right)G\left(t\right)\Delta t \quad \textbf{[S3.2a]}$$

$$M_{root}\left(t+\Delta t\right)=M_{root}\left(t\right)+\left(1-f\left(t\right)\right)\left(1-a\left(t\right)\right)G\left(t\right)\Delta t \quad \textbf{[S3.2b]}$$

$$M_{nod}\left(t+\Delta t\right)=M_{nod}\left(t\right)+\left(1-f\left(t\right)\right)a\left(t\right)G\left(t\right)\Delta t \quad \textbf{[S3.2c]}$$

Both the carbon-limited and the nitrogen-limited growth rates are linear functions of how much growth is allocated to the shoot (*f*), but non-linear functions of allocation to nodules (*a*) (since *f* is a non-linear function of *a*). Therefore, if roots and nodules provide equal amounts of nitrogen and use equal amounts of carbon per gram tissue ($m = \theta c_N$ and $d_{nod} = d_{root}$), both growth rates are independent of allocation to nodules (*a*) and a linear function of allocation to the shoot (*f*). The carbon-limited growth rate increases as more resources are allocated to the shoot (larger *f*), while the nitrogen-limited growth rate decreases (Fig. 1*C*). The maximum growth rate is achieved when carbon- and nitrogen-limited growth rates are equal, and this yields the optimal value of allocation to the shoot (*f*).

If nodules provide more nitrogen than roots without respiring more carbon, increasing allocation to nodules (*a*) increases the nitrogen-limited growth rate while the carbon-limited growth rate is unaffected. The maximum growth rate is achieved when carbon and nitrogen are equally limiting, and is highest when all resources are allocated to nodules ($a = 1$) (Fig. 1*C*). Of course, providing more nitrogen than roots without respiring more carbon is unlikely. Instead, increasing allocation to nodules (*a*) increases the nitrogen-limited growth rate, but decreases the carbon-limited growth rate, as nodules respire more carbon than roots (Fig. S1*A*). As long as the increase in the nitrogen-limited growth rate increases the overall growth rate more than the decrease in the carbon-limited growth rate, optimal allocation remains allocating all resources to nodules ($a = 1$). But there comes a point where the carbon-limited growth rate is decreased so much by allocating to nodules, that the maximum growth rate is achieved by ceasing allocation to nodules ($a = 0$) (Fig. S1*A*). The maximum growth rate always occurs at the extreme ends of allocation to nodules ($a = 0$ or $a = 1$), so that optimal allocation to nodules is all or nothing. We can express the point where plants should switch from allocating to nodules ($a = 1$) to allocating to roots ($a = 0$) in mathematical terms:

Invest in nodules if:

$$G_N(t+\Delta t)_{a=1} > G_N(t+\Delta t)_{a=0} \quad \textbf{[S3.3]}$$

(Since the optimal value for allocation to the shoot (*f*) is already known, carbon-limited and nitrogen-limited growth rates are equal ($G_C = G_N$) at all times, so either the carbon-limited or the nitrogen-limited growth rate can be considered for further derivations.)

Substituting Eq. **S3.2** into Eq. **S3.1** into Eq. **S3.3** gives:

Invest in nodules if:

$$m\left(1-f_{a=1}\right)>\theta c_N(1-f_{a=0}) \qquad \textbf{[S3.4]}$$

Substituting for allocation to shoots (*f*) (Eq. **S1.5**) and rearranging yields the optimal allocation to nodules (*a*):

Invest in nodules (*a* = 1) if:

$$\frac{m}{d_{nod}+\gamma-d_{leaf}}>\frac{\theta c_N}{d_{root}+\gamma-d_{leaf}} \qquad \textbf{[S3.5]}$$

Derivation of optimal allocation to nodules (*a*) for multiple rhizobial strains. A plant will only invest in a rhizobial strain if it provides more nitrogen or does so more efficiently than the root, and thus meets the condition in Eq. **1** (main text). If two different strains meet this requirement, then the plant could choose to invest in one or both (therefore; $a_A + a_B = 1$, so that $a_A = 1 - a_B$). Optimal allocation to two rhizobial strains (the values of parameter a_A and a_B) is found by considering the carbon-limited (G_C) and nitrogen-limited (G_N) growth rates at time $t = \Delta t$. Growth at time $t = \Delta t$ in the case of two rhizobial strains is defined as:

$$G_C\left(t+\Delta t\right)=\gamma M_{leaf}\left(t+\Delta t\right)-d_{leaf}M_{leaf}\left(t+\Delta t\right)-d_{root}M_{root}\left(t+\Delta t\right)- d_{nod,A}M_{nod,A}\left(t+\Delta t\right)-d_{nod,B}M_{nod,B}\left(t+\Delta t\right) \quad \textbf{[S4.1a]}$$

$$G_N\left(t+\Delta t\right)=\rho\theta M_{root}\left(t+\Delta t\right)c_N+\rho m_A M_{nod,A}\left(t+\Delta t\right)+\rho m_B M_{nod,B}\left(t+\Delta t\right) \quad \textbf{[S4.1b]}$$

in which

$$M_{leaf}\left(t+\Delta t\right)=M_{leaf}\left(t\right)+f\left(t\right)G\left(t\right)\Delta t \quad \textbf{[S4.2a]}$$

$$M_{root}\left(t+\Delta t\right)=M_{root}\left(t\right)+\left(1-f\left(t\right)\right)\left(1-a_A-a_B\right)\left(t\right)G\left(t\right)\Delta t \quad \textbf{[S4.2b]}$$

$$M_{nod,A}\left(t+\Delta t\right)=M_{nod,A}\left(t\right)+\left(1-f\left(t\right)\right)a_A\left(t\right)G\left(t\right)\Delta t \quad \textbf{[S4.2c]}$$

$$M_{nod,B}\left(t+\Delta t\right)=M_{nod,B}\left(t\right)+\left(1-f\left(t\right)\right)a_B\left(t\right)G\left(t\right)\Delta t \quad \textbf{[S4.2d]}$$

For a given strain (A) with nitrogen fixation rate (m_A) and efficiency ($e_A = d_{nod,A} / m_A$), the growth rate at time $t = \Delta t$ is a non-linear function of the nitrogen fixation and respiration rates of strain B, and allocation to strain B (Fig. S1*B, C*). When strain A and strain B respire equal amounts of carbon per gram nitrogen fixed, overall growth rate is highest when allocating all resources to strain A (if strain A fixes more nitrogen), or all resources to strain B (when strain B fixes more nitrogen). The overall growth rate is independent of resource allocation when the strains provide equal amounts of nitrogen (strains are indistinguishable) (Fig. 1*E*). If strain B respires more carbon per gram nitrogen fixed than strain A, then allocation to strain B only results in higher growth rates if strain B provides considerably more nitrogen than strain A (Fig. S1*C*). Again, growth rates are highest at extreme ends of allocation, so by either allocating all resources to strain A, or to strain B. Analogously to comparing roots with nodules, this yields a criterion to choose between strain A and strain B.

Invest in strain B if:

$$G_N(t+\Delta t)_{a_A=0\ \&\ a_B=1}) > G_N(t+\Delta t)_{a_A=1\ \&\ a_B=0} \quad \textbf{[S4.3]}$$

Substituting Eq. **S4.2** into Eq. **S4.1** into Eq. **S4.3** leads to:

Invest in strain B if ($a_A = 0$ and $a_B = 1$):

$$m_B(1-f_{a_A=0\ \&\ a_B=1}) > m_A(1-f_{a_A=1\ \&\ a_B=0}) \quad \textbf{[S4.4]}$$

Substituting for allocation to shoots (*f*) (Eq. **S1.6**) and rearranging yields the optimal allocation to nodules strain B:

Invest in strain B if ($a_A = 0$ and $a_B = 1$):

$$\frac{m_B}{d_{nod,B}+\gamma-d_{leaf}} > \frac{m_A}{d_{nod,A}+\gamma-d_{leaf}} \quad \textbf{[S4.5]}$$

Optimal allocation means that the plant allocates all resources to either strain A or to strain B. If strain B is the better choice, the same argument can be made for comparing strain B with strain C. Therefore, given a soil nitrogen concentration, there will always be a single rhizobial strain to which all resources should be allocated for maximum growth.

Cloning methods and results. OPS1474 (previously named LMB827) was originally intended to be a *fixC* deletion mutant isolated by conjugation into Rlv3841 of plasmid pLMB878 (Fig. S3*A*). However, the plasmid was instead inserted in between *nifA* and *fixX*, leaving *fixC* untouched, as was shown by whole genome sequencing (Fig. S3*B*). Instead of disrupting *fixC,* read through from the *NifA* dependent promoter upstream of *fixA* is prevented. This will disrupt transcription of genes downstream of *fixB* (i.e. *fixC, fixX* and *nifA*) and prevent full auto-upregulation of the operon by *NifA* under N_2-fixing conditions, hence the reduced fixation phenotype.

Strains marked with *lux* genes, fluorescent proteins (sfGFP (3) or mCherry3 (4)), or the pJP2 control plasmid were made using marker plasmids (Table S3). Plasmids were added to strains either by mini-Tn7 insertion into the chromosome (5) using pTNS3 (6), or on a stable plasmid via triparental mating with the assistance of *pRK2013* (7). The combinations of recipient strain, donor plasmid and method are detailed in (Table S2).

To make the plasmid expressing *lux* genes under the *nifH* promoter (pLMB640), the *nifH* promoter from Rlv3841 was amplified by PCR using primers pr1348 (TTTGGATCCAGATCAAGAA CCGTATCCTG) and pr1349 (TTTGGTACCAATCAATTATCCCGTCGTTG). The 934 bp PCR product of pr1348 and pr1349 was purified and digested with BamHI and KpnI (pr1348 has a BamHI site added at the 5' end for cloning and pr1349 has a KpnI site for cloning) and ligated into BamHI KpnI digested pIJ11268 (8). The resulting plasmid pLMB640 is 17,176 bp with the *nifH* promoter expressing *luxCDABE*.

Characteristics of Fix$^+$, Fix^{int} and Fix$^-$ in single inoculations. In single inoculations, acetylene reductions revealed that Fix^{int} (OPS1474) fixed nitrogen at reduced rates compared to Fix$^+$. Fix^{int} fixed 0.011, 95% CI [0.007, 0.014] µmol ethylene nodule^{-1} hour^{-1} less than Fix$^+$ at 27 days post inoculation (dpi), a reduction of 74% (Fig. S4*A*). When expressed per plant (thus taking into account the total number of nodules), Fix^{int} fixed 0.83, 95% CI [0.43, 1.23] µmol ethylene plant^{-1} hour^{-1} less than Fix$^+$ at 27 dpi, a reduction of 48%.

The nodules on these plants were counted and analyzed as a function of strain using a generalized linear model with a quasipoisson distribution. Plants inoculated with Fix^{int} have a similar number of nodules to those inoculated with Fix$^-$ (mean Fix^{int} 263, mean Fix$^-$ 261, ns difference with risk ratio (RR) 0.99, 95% CI [0.75, 1.30]), and more nodules than plants inoculated with Fix$^+$ (mean Fix$^+$ 125, RR 0.47, 95% CI [0.32, 0.69]) (Fig. S4*B*). The shoot biomasses of these plants was obtained by drying the shoots in a 70°C incubator for 14 days, then weighing the shoots. Shoot biomasses were analyzed as a function of strain using a linear model. Plants inoculated with Fix^{int} have an intermediate dry shoot biomass (mean 0.42 g), more per plant than Fix$^-$ (0.15 g more, 95% CI [0.04, 0.25]), and less per plant than Fix$^+$ (0.21 g less, 95% CI [0.10, 0.32]) (Fig. S4*C*).

Results based on randomly selected nodules. In single inoculations, randomly selected nodules of the Fix^{int} strain averaged 1.52 mm^2, 95% CI [0.90, 2.13], which is larger than Fix$^-$ nodules (mean 0.99 mm^2, a difference of 0.53 mm^2, 95% CI [0.06, 0.99]), and smaller than Fix$^+$ nodules (mean 2.23 mm^2, a difference of 0.71 mm^2, 95% CI [0.27, 1.16]) (Fig. S5*A*). These results are in line with our findings based on measuring the most developed nodules.

In line with results based on the most developed nodules, the average size of randomly selected Fix^{int} nodules was decreased when co-inoculated with Fix$^+$ (mean 0.81 mm^2), compared to when co-inoculated with itself (mean 1.29 mm^2) (a difference of 0.48 mm^2, 95% CI [0.29, 0.66]). Co-inoculation with the Fix$^-$ strain led to slightly larger nodules of the Fix^{int} strain (mean 1.57 mm^2) as compared to when co-inoculated with itself (a difference of 0.28 mm^2, 95% CI [0.09, 0.46]) (Fig. S5*B*). For comparison, average nodule sizes of randomly selected nodules of the Fix$^-$ and Fix$^+$ strain are shown (Fig. S5*B*, orange points). The difference in size between Fix$^+$ and Fix^{int} was larger

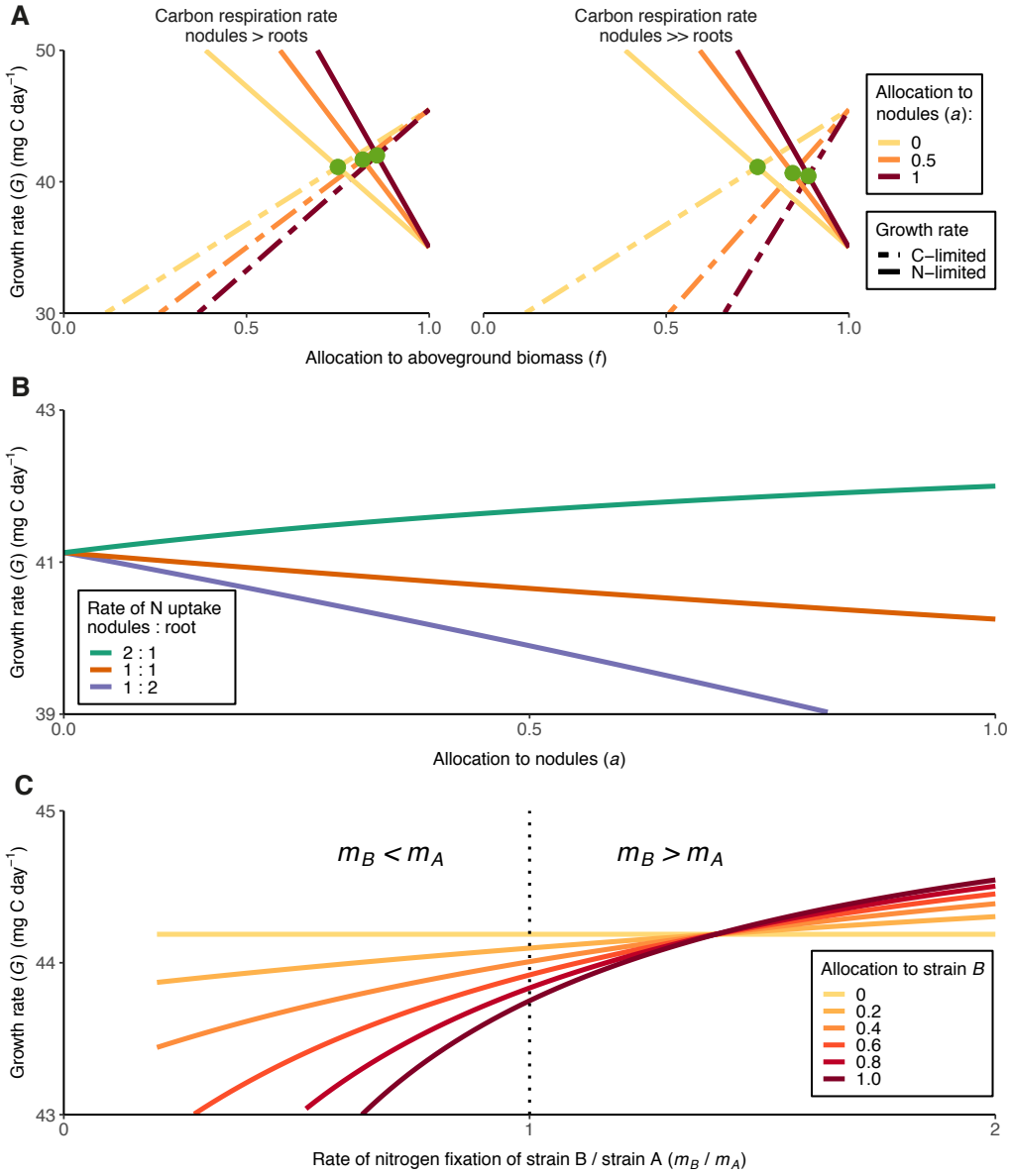
when they were co-inoculated as compared to in single inoculations (significant interaction between strain and inoculation type (singly or co-inoculated), $F_{1,31}$ = 12.8457, p = 0.0011).

Light microscopy methods and results. In order to obtain light microscopy images of nodules in co-inoculation experiments, a Fix^{int} strain was co-inoculated with Fix^{-} or Fix^{+} strains. The Fix^{int} strain was GFP-marked (OPS1478) and co-inoculated with mCherry-marked Fix^{-} or Fix^{+} strains (OPS0930 or OPS0900 respectively), or mCherry-marked (OPS1479) and co-inoculated with GFP-marked Fix^{-} or Fix^{+} strains (OPS0929 or OPS0904 respectively). Plants were inoculated with 1:1 mixtures of the strains at a total density of 1 x 10^4 cells per pot. Per co-inoculated strain (Fix^{-} or Fix^{+}), two replicates (GFP-marked Fix^{int} strain with mCherry-marked co-inoculated strain and vice versa) and one water control were grown in a fully randomized design.

At harvest (27 dpi), plants were gently removed from the pots and shaken to remove any clinging substrate. Roots were carefully laid out on 120 x 120 mm^2 plates so all nodules were visible, placed on a blue-light transilluminator (VWR), and photographed using an orange UV filter. Three GFP and three mCherry nodules per plant were selected based on their fluorescence, using a Leica M165 fluorescence camera to distinguish between markers (GFP was detected using a GFP3 filter with 475-495 nm excitation and 505-540 nm barrier, and mCherry using a mCherry filter with 540-580 nm excitation and 593-668 nm barrier). Selected nodules were harvested by cutting the adjoining root with a scalpel, so as not to damage the nodules, and kept on ice before further processing.

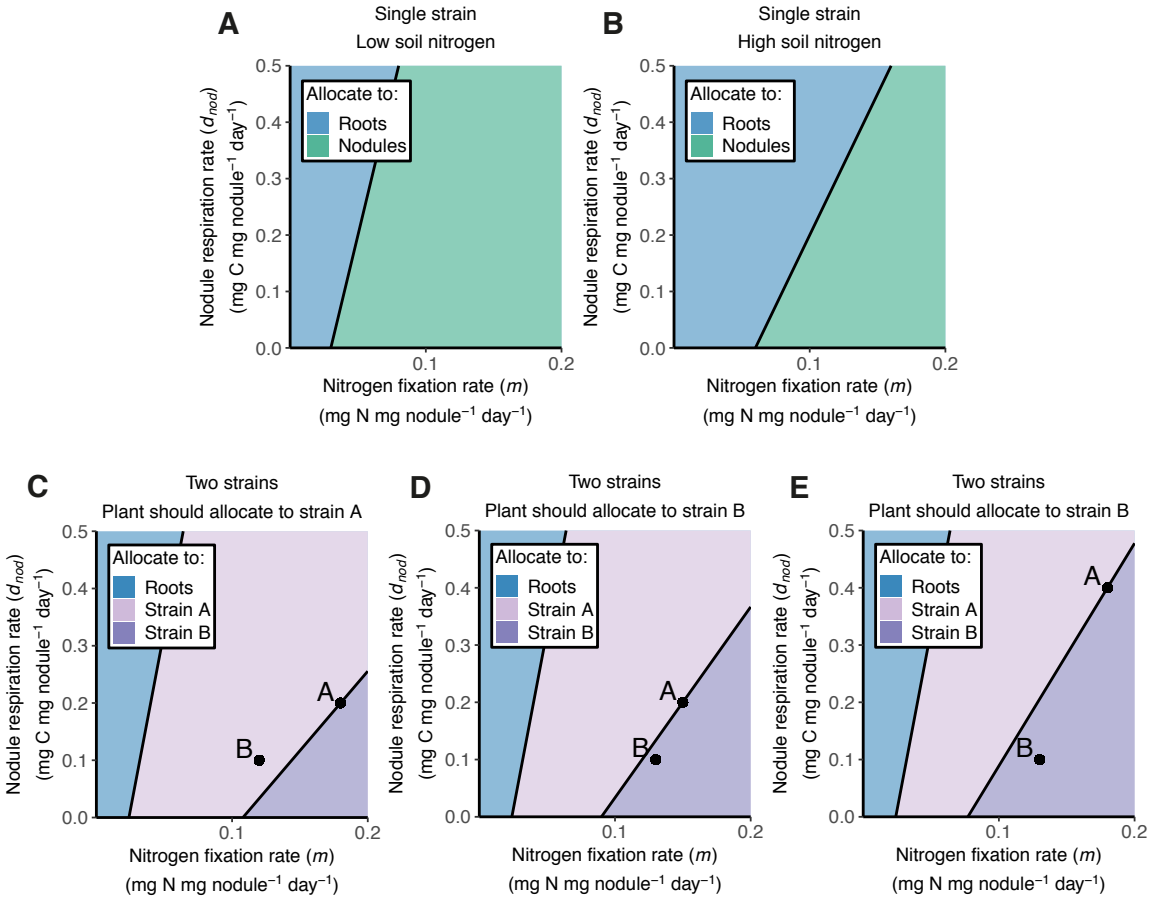
Nodules were sterilized by immersion in 2% sodium hypochlorite for 1 minute and then washed six times in sterile water to remove any traces of bleach. Using sterile tweezers and a razor blade, nodules were then sliced in half along the long axis (perpendicular to the root) and placed in an Eppendorf tube with 0.5 ml of fixative (2.5% glutaraldehyde in 0.1 M filter sterilized (0.22 µm) sodium cacodylate (pH 7)). Nodules were embedded in resin then stained for light microscopy as in (2). At least three nodules per treatment were observed, with representative nodules displayed in Fig. S6.

Fig. S1.



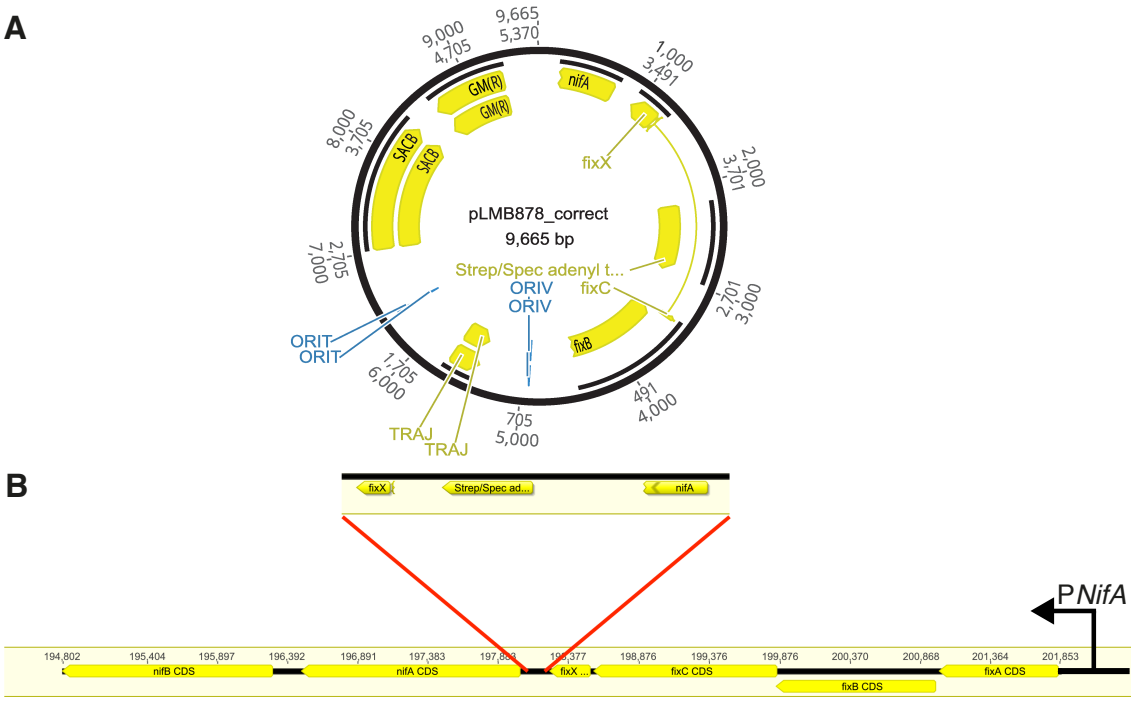
Optimal allocation to nodules is always 'all or nothing', shown for cases in which nodules respire more carbon than roots (*A, B*) or strain B respires more carbon than strain A (*C*). The overall growth rate is defined as the minimum of carbon- and nitrogen-limited growth rates, and is highest where carbon and nitrogen are equally limiting (green dots), at the optimal value of allocation to the shoot. Growth rates increase with increasing allocation to nodules if they provide more nitrogen than roots, unless they also respire too much carbon (*A*). When nodules respire more carbon than roots, the plant should only invest in them if they provide considerably more nitrogen than roots (*B*). Similarly, in the case of two strains, the plant should only invest in a strain B which respires more carbon than strain A if it also provides considerably more nitrogen (*C*). (Note that lines in the case of two strains only start beyond the rate of nitrogen fixation at which nodules rather than roots should be invested in). Since the highest growth rates are always at either $a = 0$ or at $a = 1$, optimal allocation to nodules is all or nothing.

Fig. S2.



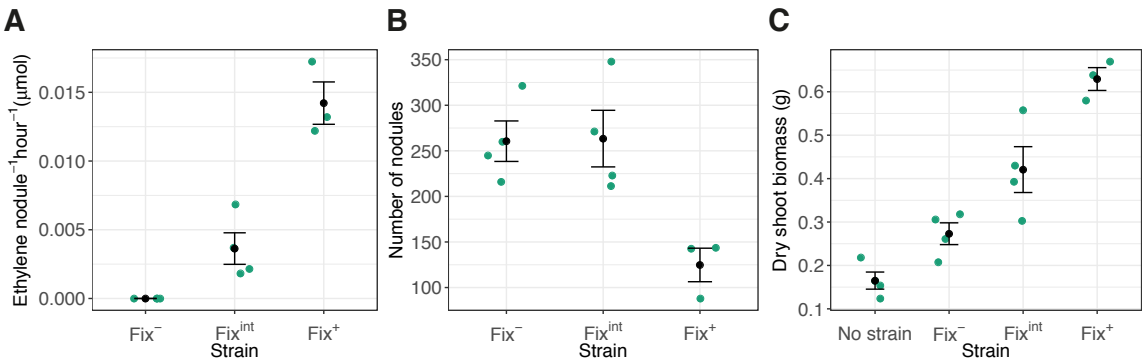
Model prediction: investment in rhizobial strains is context-dependent. Whether plants should invest in roots versus nodules depends on rhizobial nitrogen fixation rates, carbon demands, and the degree of nitrogen limitation experienced by the plant. The slope of the line is given by Eq. 1 and is steeper for more nitrogen-limited plants (*A*), which should invest in less effective and efficient strains than plants which have more access to soil nitrogen (*B*). When two strains are present which both meet the condition of Eq. 1, the model predicts that the plant should invest only in the best available strain. Given a strain A, the plant should only allocate resources to a second strain B if it is more effective or efficient, or if strain A is unavailable. For example, the plant should invest in strain A and sanction strain B (less effective and efficient) when both strains are present (*C*). However, the plant should invest in strain B if strain A is a less effective (*D*) or less efficient (*E*) strain. The slope of the line (Eq. 2) is steeper for less carbon-limited plants, which should invest in less efficient strains.

Fig. S3.



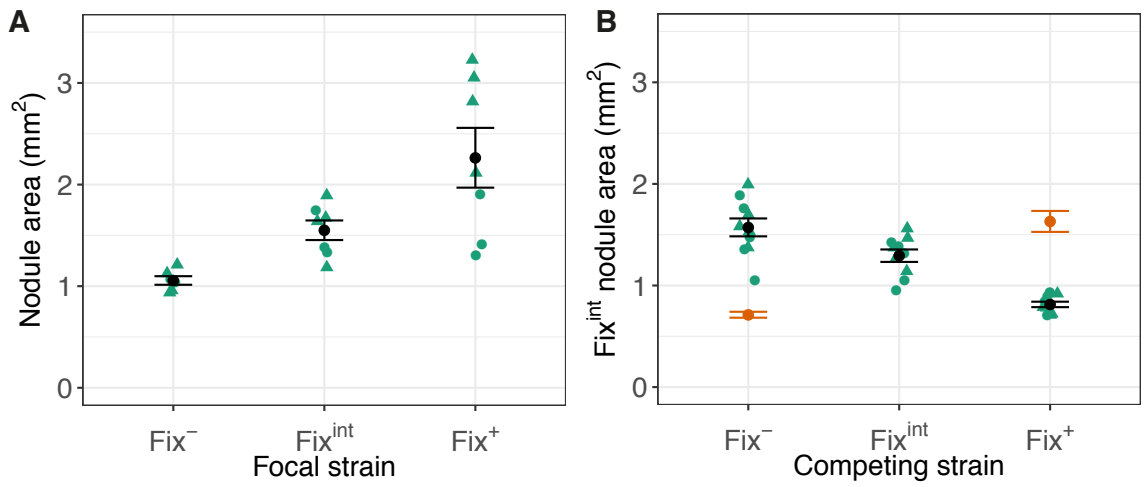
Genomic structure of strain OPS1474. Plasmid pLMB878 (*A*) was designed for a *fixC* deletion but the plasmid was instead inserted in between *nifA* and *fixX*, leaving *fixC* untouched (*B*). Instead of disrupting *fixC,* read through from the NifA-dependent promoter upstream of *fixA* is prevented (P*nifA*, directionality indicated by an arrow at the right of *(B)*). This will disrupt transcription of genes downstream of *fixX* (i.e. *nifA* and *nifB*) and prevent full auto-upregulation of the operon by NifA under N_2-fixing conditions, hence the reduced fixation phenotype.

Fig. S4.



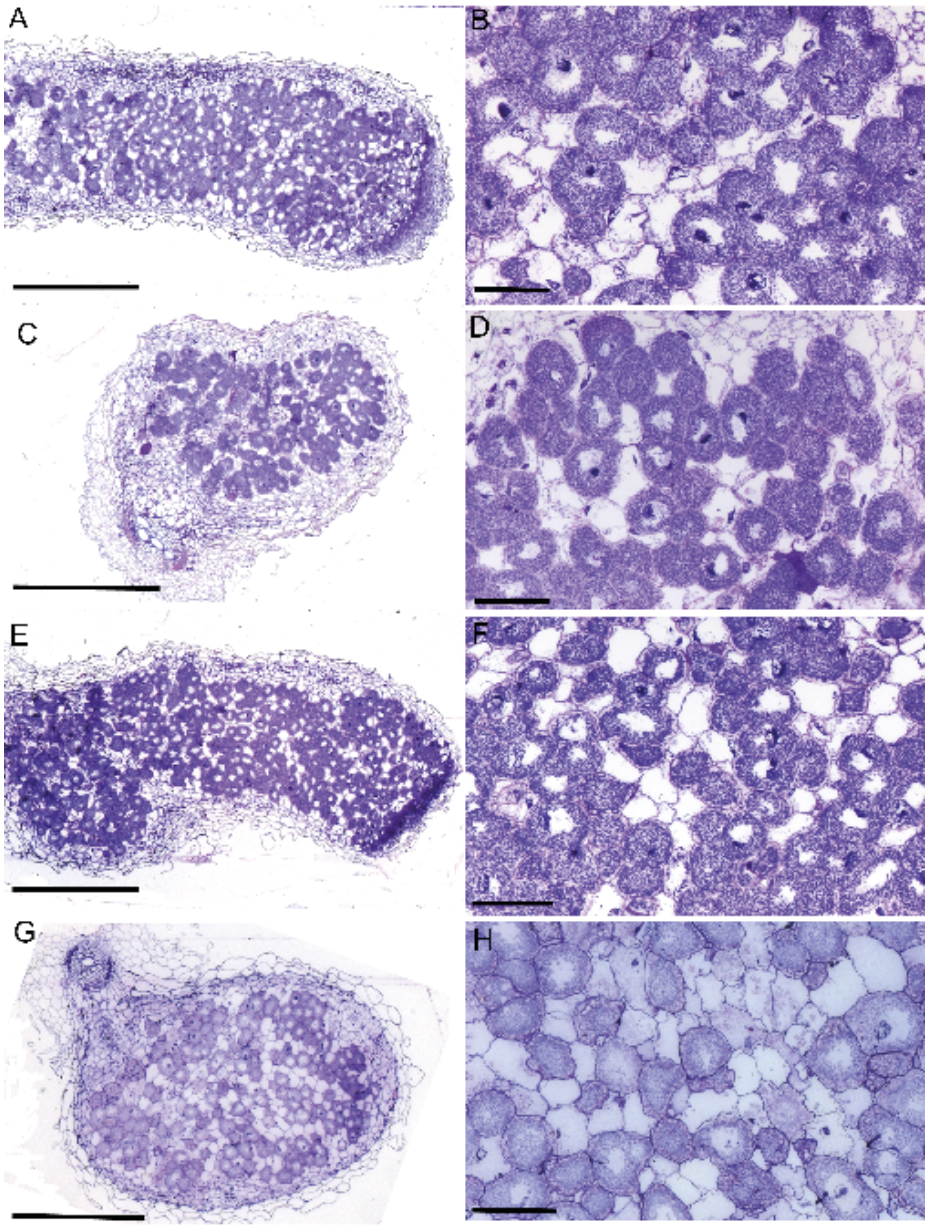
Nitrogen fixation rates (*A*), number of nodules (*B*) and dry shoot biomass (*C*) of single inoculated plants. Pea roots were inoculated with single inoculations of non-fixing (Fix^{-}), intermediate-fixing (Fix^{int}), and fixing (Fix^{+}) strains. Fix^{int} has an intermediate rate of nitrogen fixation at 27 dpi (*A*). Plants inoculated with Fix^{-} or Fix^{int} form more nodules than plants inoculated with Fix^{+} (*B*) and have an intermediate dry shoot biomass (*C*). Points represent an average of three technical replicates per independent replicate plant (*A, B*), or a single measurement per independent replicate plant (*C*). Black points indicate the overall mean with one standard error. Plants were grown for 27 days. (*A*-*C*) n = 3-4.

Fig. S5.



Nodule size of an intermediate-fixing strain is conditional on which other strains are present on the plant. Pea roots were inoculated with different combinations of non-fixing, (Fix$^-$), intermediate-fixing (Fix^{int}), and fixing (Fix$^+$) strains. In single inoculations (*A*), and in co-inoculations (*B*), Fix^{int} nodules were intermediate in size. Points represent an average of ten randomly chosen nodules per strain and per independent replicate plant. Symbols indicate data from independent experiments. Black points indicate the overall mean with one standard error, orange points indicate this for competitor nodules on the same plants. Plants were grown for 27-28 days. (*A*) n = 6-7, (*B*) n = 10.

Fig. S6.



Light microscopy images of nodules in co-inoculations. Pea roots were inoculated with different combinations of a non-fixing strain (Fix^-), an intermediate-fixing strain (Fix^{int}), and a fixing strain (Fix^+). When Fix^{int} and Fix^+ were co-inoculated (*A-D)*, Fix^+ nodules had an active meristem (*A*) and healthy cells (*B*) at harvest. In contrast, Fix^{int} nodules were senescing prematurely, appeared to have no active meristem and had a much smaller infected zone III (*C*), even though cells looked fairly normal (*D*). When Fix^{int} and Fix^- nodules were co-inoculated (*E-H*), Fix^{int} nodules did maintain an active meristem (*E*) with healthy cells (*F*), while Fix^- nodules were senescing prematurely (*G*) and cells were breaking down (*H*). Scale bars indicate 500 µm (*A*, *C*, *E*, *G*) and 50 µm (*B*, *D*, *F*, *H*). Note that cells shown are bacteroids (which cannot reproduce), not the undifferentiated bacteria which determine fitness.

Table S1. Parameter values used for model implementation.

Parameter	Description	Value and/or range	Unit	Reference
γ	C uptake rate (photosynthesis)	0.5	mg C mg shoot^{-1} d^{-1}	(1, 9)
ρ	C:N ratio	7.0	dimension-less	(1)
θ	Soil exploration rate of root	2	cm^3 mg^{-1} d^{-1}	(1, 10)
c_N	N concentration in soil	0 – 0.05	mg cm^{-3}	(1) and experimental conditions.
θc_N	N uptake rate per gram root	0 – 0.1	mg N mg root^{-1} d^{-1}	Based on θ and c_N.
m	N fixation rate	0 – 1	mg N mg nodule^{-1} d^{-1}	Up to ten times as much nitrogen per mg tissue as roots.
d_{leaf}	Shoot respiration rate	0.2	mg C mg shoot^{-1} d^{-1}	Similar to root respiration (9).
d_{root}	Root respiration rate	0.2	mg C mg root^{-1} d^{-1}	± 2 mg C per mg N uptake (11–13).
d_{nod}	Nodule respiration rate	0 – 10 ($d_{nod} = em$)	mg C mg nodule^{-1} d^{-1}	Up to efficiency (e) of ten mg C per mg N fixation (estimated average ± 6 (14, 15))
M_{seed}	Seed mass	200	mg	Experimental conditions
f	Fraction of growth allocated to shoot	0 – 1	dimension-less	Analytically derived / numerically tested
a	Fraction of belowground growth allocated to nodules	0 – 1	dimension-less	Analytically derived / numerically tested

Table S2. Strains used in this study.

Strain	Description	Antibiotic resistance*	Reference
LMB640	RU3940[pJP2] (Rlv3841 *nifH::Ω* Sp^r with pJP2)	St, Sp, Tc	(16)
OPS0365	Rlv3841 *gusA nifH::Ω* Sp^r	St, Sp	(17)
OPS0366	Rlv3841 *celB nifH::Ω* Sp^r	St, Sp	(17)
OPS0900	Rlv3841[pOPS0380] (Rlv3841 with mCherry3 under *nifH* promoter)	St, Nm	(18)
OPS0904	Rlv3841[pOPS0379] (Rlv3841 with sfGFP under *nifH* promoter)	St, Nm	(18)
OPS0929	RU3940[pOPS0379] (Rlv3841 *nifH::Ω* Sp^r with sfGFP under *nifH* promoter) (triparental)	St, Sp, Nm	This work
OPS0930	RU3940[pOPS0380] (Rlv3841 *nifH::Ω* Sp^r with mCherry3 under *nifH* promoter) (triparental)	St, Sp, Nm	This work
OPS1339	Rlv3841 with pOPS0743 used to insert sfGFP under J23104 promoter (Tn7)	St, Gm	This work
OPS1341	Rlv3841 with pOPS1015 used to insert mCherry3 under J23104 promoter (Tn7)	St, Gm	This work
OPS1474	*Ω* Sp^r resistant insertion between *nifA* and *fixX* of Rlv3841, intermediate fixation phenotype (previously named LMB827)	St, Sp	This work
OPS1478	OPS1474[pOPS0379] (intermediate fixer with sfGFP under *nifH* promoter) (triparental)	St, Sp, Nm	This work
OPS1479	OPS1474[pOPS0380] (intermediate fixer with mCherry3 under *nifH* promoter) (triparental)	St, Sp, Nm	This work
OPS2136	OPS1474[pLMB714] (intermediate fixer with C4-dicarboxylates biosensor) (triparental)	St, Sp, Tc	This work
OPS2137	OPS1474[pLMB687] (intermediate fixer with sucrose biosensor) (triparental)	St, Sp, Tc	This work
OPS2268	OPS1474 (intermediate fixer) with pOPS1526 used to insert sfGFP under J23104 promoter (Tn7)	St, Sp, Gm	This work
OPS2269	OPS1474 (intermediate fixer) with pOPS1531 used to insert mCherry3 under J23104 promoter (Tn7)	St, Sp, Gm	This work
OPS2270	RU3940 (Rlv3841 *nifH::Ω* Sp^r) with pOPS1526 used to insert sfGFP under J23104 promoter (Tn7)	St, Sp, Gm	This work
OPS2271	RU3940 (Rlv3841 *nifH::Ω* Sp^r) with pOPS1531 used to insert mCherry3 under J23104 promoter (Tn7)	St, Sp, Gm	This work
OPS2272	RU1416 (Rlv3841[pJP2]) with pOPS1531 used to insert mCherry3 under J23104 promoter (Tn7)	St, Gm, Tc	This work
OPS2273	LMB640 (Rlv3841 *nifH::Ω* Sp^r with pJP2) with pOPS1531 used to insert mCherry3 under J23104 promoter (Tn7)	St, Sp, Gm, Tc	This work
OPS2278	OPS2137 (intermediate fixer with sucrose biosensor) with pOPS1526 used to insert sfGFP under J23104 promoter (Tn7)	St, Sp, Gm, Tc	This work
OPS2313	OPS2136 (intermediate fixer with C4-dicarboxylates biosensor) with pOPS1526 used to insert sfGFP under J23104 promoter (Tn7)	St, Sp, Gm, Tc	This work
OPS2314	OPS2268[pLMB640] (intermediate fixer with pOPS1526 used to insert sfGFP under J23104 promoter and with P*nifH* biosensor) (triparental)	St, Sp, Gm, Tc	This work
Rlv3841	St^r derivative of *R. leguminosarum* bv. *viciae* strain 300	St	(19)
Rlv3841 *gusA*	Rlv3841 with *gusA* marker	St	(20)

RU1416	Rlv3841[pJP2]	St, Tc	(21)
RU3940	Rlv3841 *nifH::Ω* Sp^r, non-fixing phenotype	St, Sp	(17)

*Gm = gentamicin (20 μg ml^{-1}), Nm = neomycin (40 μg ml^{-1}), Sp = spectinomycin (100 μg ml^{-1}), St = streptomycin (500 μg ml^{-1}), Tc = tetracycline (5 μg ml^{-1}).

Table S3. Plasmids used in this study.

Plasmid	Description	Antibiotic resistance*	Reference
pIJ11268	Broad host range; promoterless *luxCDABE* derived from pJP2	Amp, Tc	(8)
pJP2	Stable broad host range cloning vector	Tc	(22)
pLMB640	P*nifH* biosensor, pIJ11268 with *nifH* promoter cloned in front of *luxCDABE*	Tc	This work
pLMB687	Sucrose biosensor, pIJ11268 with sucrose transporter promoter cloned in front of *luxCDABE*	Tc	(23)
pLMB714	C4-dicarboxylates biosensor, pIJ11268 with *dctA* promoter cloned in front of *luxCDABE*	Tc	(23)
pLMB878	*fixC::Ω* Sp^r in pJQ200SK	Gm, Sp	This work
pOPS0379	Reporter plasmid constructed with Rlv3841P*nifH* (pOGG082) sfGFP (pOGG037) and T-pharma (pOGG003) assembled in pOGG026	Km	(24)
pOPS0380	Reporter plasmid constructed with promoter Rlv3841P*nifH* (pOGG082), mCherry3 (EC15071) and T-pharma (pOGG003) assembled in pOGG026	Km	(18)
pOPS0743	sfGFP under J23104 promoter in a pUC18T-miniTn7T-Gm vector for Tn7 insertion	Amp, Gm	This work
pOPS1015	mCherry3 under J23104 promoter in a pUC18T-miniTn7T-Gm vector for Tn7 insertion	Amp, Gm	This work
pOPS1526	sfGFP under J23104 promoter in a pUC18R6KT-miniTn7T-Gm vector for Tn7 insertion	Amp, Gm	This work
pOPS1531	mCherry3 under J23104 promoter in a pUC18R6KT-miniTn7T-Gm vector for Tn7 insertion	Amp, Gm	This work
pRK2013	Helper plasmid for triparental conjugation	Km	(25)
pTNS3	Plasmid expressing *tnsABCD* from *P1* and P_{lac}	Amp	(6)
pUC18T-miniTn7T-Gm	Plasmid for Tn7 insertion into Gm sensitive bacteria	Amp, Gm	(5, 6)
pUC18R6KT-miniTn7T-Gm	Plasmid for Tn7 insertion into Gm sensitive bacteria	Amp, Gm	This work

*Amp = ampicillin (100 µg ml^{-1}), Gm = gentamicin (10 µg ml^{-1}), Km = kanamycin (20 µg ml^{-1}), Sp = spectinomycin (50 µg ml^{-1}), Tc = tetracycline (10 µg ml^{-1}).

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