The regulation of the legume symbiosis with nitrogen-fixing bacteria

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DECLARATION OF AUTHORSHIP

I declare that all parts of this thesis are my own work, except for some data in Chapters 3 & 5 which are the work of contributing researchers: Beatriz Jorrin & Rachel Andres. Details of these contributions are given in the authorship declaration forms that follows these chapters.

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

Thomas J Underwood

When someone was down you helped, not out of altruism, but an enlightened selfishness.

-Richard Flanagan, Question 7

ABSTRACT

Leguminous plants form a symbiotic relationship with nitrogen-fixing bacteria called rhizobia. These rhizobia are housed within specialised root structures called nodules. Inside these nodules the rhizobia differentiate into nitrogen-fixing bacteroids and convert atmospheric N_2 to ammonia which is provided to the host plant in return for photosynthetically derived carbon in the form of malate.

Rhizobia expend large amounts of energy on fixation, therefore there is a fitness benefit to fixing less nitrogen and using the energy for replication. This could create a selection pressure for the rhizobia to 'cheat'. However, it has been shown previously that legumes conditionally sanction nodules containing cheating strains. I have shown that these punishments cause a drop in the number of bacteroids and premature senescence of the whole nodule. Sometimes nodules are inhabited by multiple strains of varying fixation effectiveness. I have demonstrated that the host plant is unable to differentiate between these strains and instead sanctions at the whole nodule level.

Avenues do exist for 'cheating' bacteria to thrive. I showed that the severity of sanctioning is dependent on the ratio of cheats to non-cheats. Therefore, if a cheat occupies most nodules they can evade sanctions. I also showed that the application of sanctions is based on a global comparison of nodule effectiveness and that host plants can differentiate between small variations in effectiveness.

Finally, I investigated the mechanism that regulates sanctioning and through RNA sequencing discovered several candidate regulators. Follow up experiments suggested that the plant hormone ABA may be a regulator.

These findings improve our understanding of how legumes regulate their symbiotic relationships with a variety of strains. They also provide new insight into the mechanism controlling this regulation. This knowledge is fundamentally important for the exploitation of this symbiosis in reducing reliance on artificial fertilisers.

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
ARA	Acetylene reduction assay
AMF	Arbuscular Mycorrhizal Fungi
FSC	Forward scatter
GFP	Green fluorescent protein
RNA	Ribonucleic acid
ATP	Adenosine triphosphate
LCO	Lipochitooligosaccharide
NCR	Nodule-specific cysteine rich
IRLC	Inverted repeat-lacking clade
DNA	Deoxyribonucleic acid
TCA	Tricarboxylic acid
dpi	Days post inoculation
TY	Tryptone yeast
OD	Optical density
UV	Ultra-violet
AU	Arbitrary units
CFU	Colony forming units
NS	non-significant
SE	Standard error
GM	Gentamicin
Spec	Spectinomycin
Strep	Streptomycin
BFP	Blue fluorescent protein
w/v	Weight per volume

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1 Introduction

1.1 THE NITROGEN CRISIS

A primary goal of modern agriculture is to ensure food security. There have been numerous major advancements in this pursuit over the last hundred years. Most notably the green revolution of the late 20th century was a key turning point as the pace of population growth threatened to outstrip the growth in agricultural yields (John & Babu, 2021). However, once again the margin for food security is becoming thinner and in a number of areas around the globe food security is now no longer ensured and/or was never achieved to begin with.

The key difference between this moment of crisis and the green revolution is the simultaneous need to curb the emission of greenhouse gases and to stave off the worst effects of climate change. This is of particular relevance to the use of artificial fertilisers.

Despite all the major advancements in agriculture the basic use and design of fertilisers have remained essentially unchanged. While initially made exclusively through the harvesting of guano and eventually through the Haber-Bosch process (Hailes, 2023) the goal has always been to provide crops with a large quantity of essential molecules, namely compounds containing phosphorous and nitrogen, in an easily spreadable form throughout a field.

The large-scale manufacturing of artificial fertilisers using the Haber-Bosch process which has increased rapidly over the last decades to keep pace with food production

has become a major contributor to climate change. The fertiliser supply chain running from production through transport and application made up 2.1% of global greenhouse gas emissions in 2018, representing 1.13 gigatonnes of CO_2 emissions (Menegat et al., 2022). The application of artificial fertiliser also results in the emission of nitrous oxide (N_2O) from the fields where it is applied. N_2O is an extremely potent greenhouse gas with a warming potential approximately three hundred times that of CO_2 (Griffis et al., 2017).

The increased use of these fertilisers in fields, leading to runoff into freshwater ecosystems, has also driven eutrophication. The process by which this runoff spikes the growth of algae leading to an ‘algal bloom’. When this bloom dies as resources dwindle this drives a commensurate rise in the activity of saprophytic organisms, particularly bacteria, within the water resulting in a rapid decrease in the oxygen levels of the water and killing off other organisms (Vinçon-Leite & Casenave, 2019).

The destruction of freshwater ecosystems, coupled with the reduction in arable land driven by greenhouse gas created climate change, has only increased the pressure on food security. This has created a vicious cycle in which the increased use of fertilisers has driven climate change, lowering agricultural output thereby necessitating the use of more fertilisers. This cycle has now reached a point at which global food security is so dependent on artificial fertilisers that it is estimated that almost half of all agricultural output is derived from the Haber-Bosch process (Smil, 2002). When combined with the extreme instability in the price of fertiliser, driven by environmental and geo-political events e.g. the war in Ukraine, it becomes clear that a solution to the

ever-increasing use of artificial fertilisers is desperately needed (Arndt et al., 2023). An alternative to the use of artificial fertilisers is to explore biological sources of nitrogen.

1.2 BIOLOGICAL NITROGEN FIXATION

In order to access nitrogen in environments when nitrogen is not biologically available some bacteria have evolved or gained by horizontal gene transfer (Andrews et al., 2018), the ability to fix atmospheric N_2 and convert it into ammonia (O'Hara, 1998). This process is achieved through the use of the enzyme nitrogenase. Nitrogenase allows the reduction of N_2 into $2NH_3$ through the reductive potential of 8 electrons using 16 ATP to reach the activation energy for this reaction (Inomura et al., 2017). The enzyme is synthesised, maintained, regulated and fuelled by the activity of the *nif* and *fix* genes (Geddes et al., 2021).

The ability to fix nitrogen is found across a wide variety of bacteria, some of these are diazotrophic and are always found as free-living bacteria (Batista & Dixon, 2019). Some of these diazotrophic fixers do associate with plant roots but don't engage in a formal symbiosis. However, some of these bacteria are able to form a full symbiotic relationship with a subset of plants.

1.3 THE LEGUME – *RHIZOBIUM* SYMBIOSIS

1.3.i Diversity

The ability to form a symbiosis with nitrogen-fixing bacteria is not limited to the legumes. This symbiosis is a trait found spread across the Rosids in a paraphyletic

manner (Doyle, 2016). However, the legumes are the group in which this symbiosis is found in the most agriculturally significant plants e.g. pea, soybean, chickpea, lentil and peanut. Amongst these agricultural examples this symbiosis occurs within specialised root structures called nodules. These nodules act as a protective space for the rhizobia and provide the correct conditions for fixation (Gage, 2004).

Even within the legumes there is still a huge diversity in the nature of the symbiosis. For example, there are two major nodule types produced by legumes. These are determinate and indeterminate nodules (Popp & Ott, 2011) (Fig. 1). Determinate nodules lose meristematic activity upon maturity and contain a single interior fixing zone. In contrast indeterminate nodules have a persistent meristem. These nodules are made up of several zones making up a dividing zone at the tip of the nodule, a fixing zone in the centre and a senescent zone at the base. *Pisum sativum*, the model system used for this project, produces indeterminate nodules (Ivanova et al., 2022).

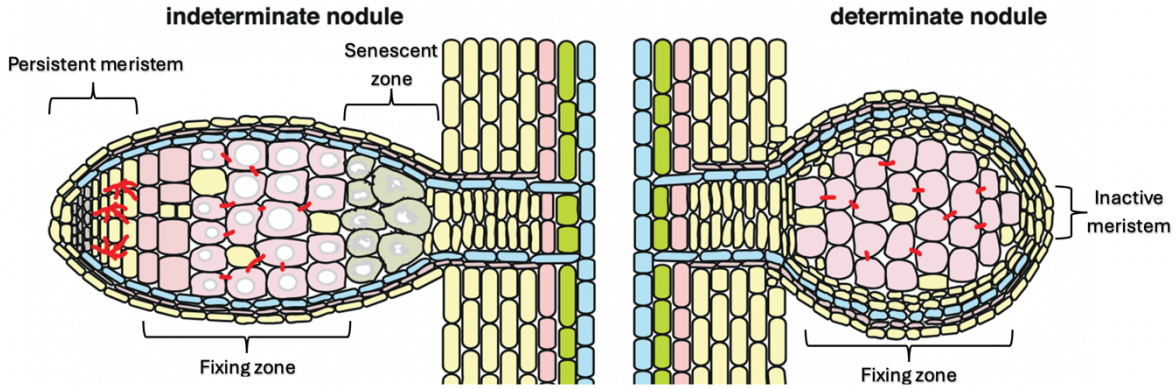

Fig. 1 Indeterminate and Determinate nodules

There are two major types of, nitrogen fixing bacteria containing, root nodules contain. Determinate nodules terminate development upon maturity and contain a single continuous fixing zone of cells. In contrast, indeterminate nodules undergo continuous development and contain discrete zones. A persistent meristem at the tip, a central fixing zone and at its base a zone of senescing cells. Adapted from Popp & Ott (2011).

1.3.ii Infection of Pisum sativum by Rhizobium leguminosarum

As discussed above there is a great deal of diversity in the legume-rhizobia symbiosis in both the legume and the rhizobial partner. When discussing the specifics of the infection and nodule process the example of the *Pisum sativum* and *Rhizobium leguminosarum* bv. viceae 3841 will be used as this is the partnership used for the studies within this thesis.

Plants exude a variety of compounds into the area surrounding their root systems (the rhizosphere). These root exudates act as a chemoattractant to rhizobia. Once near the roots the presence of exuded flavonoids induces the release of lipochitooligosaccharides (LCOs) also known as nod factors. These nod factors signal the presence of the rhizobia to the plant. LCOs bind to lysine motif receptors on the surface of root hairs triggering a signalling cascade. This reciprocal signalling and the specificity of the signals is a crucial element of the symbiosis. Allowing an organism to enter the plant carries significant risk as the organism could be pathogenic or parasitic. Therefore, this intricate signalling is designed to ensure that only a compatible symbiont enters the plant (Poole et al., 2018). The signalling cascade typically results in rhizobial attachment to root hairs which triggers root hair curling and results in the entrapment of rhizobia within the curled root hair (Oldroyd et al., 2011) (Fig. 2A).

1.3.iii Nodule formation and function

Once a rhizobium has been entrapped within a curled root hair the plant triggers specific cell wall degradation of cells within the root hair to allow the formation of the

infection thread. An infection thread is a tube running from the site of infection at the root hair down through the hair into the root proper in which the rhizobia's division drives its progression down the infection thread. At the base of the infection thread individual rhizobia are housed within infection droplets. These droplets are then endocytosed to form symbiosomes (Oldroyd et al., 2011) (Fig. 2B).

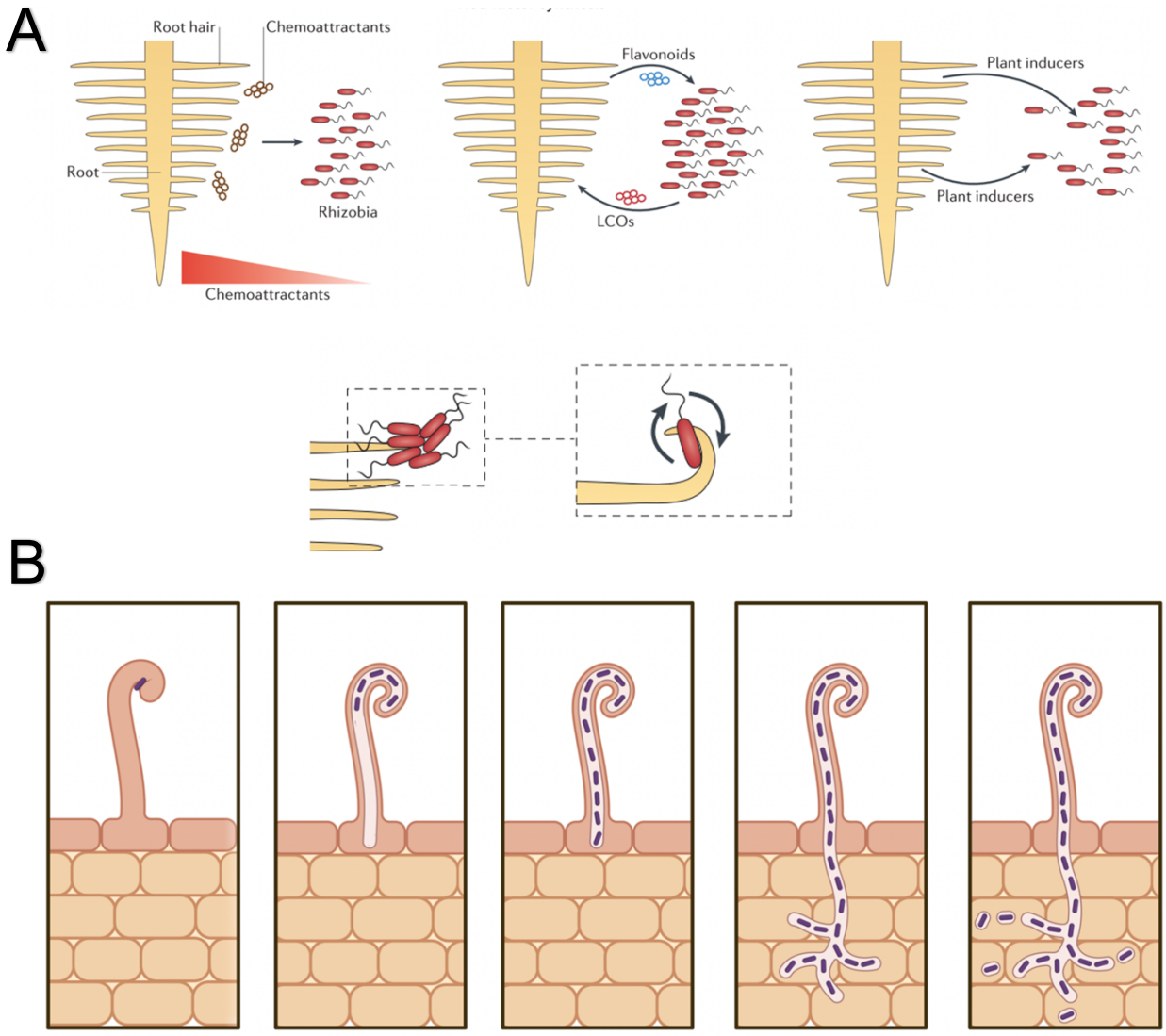

Fig.2 Rhizobial attraction, attachment and nodule infection

Rhizobia are attracted towards plant roots via chemotaxis towards plant exudates. Once in close proximity flavonoids released from the root induce expression of LCO's by the rhizobia that signal their presence to the root. A signalling cascade follows inducing the bacteria to attach to the root surface where root hair curling traps the bacteria (A). Once within the curled root hair infection thread formation is triggered. The pre-infection thread forms down the root hair through to the root proper. The rhizobia move down the infection thread by dividing. At the base of the infection thread the rhizobia are released into the root space via exocytosis within specialised plant cells called symbiosomes. Each symbiosome will contain a single bacterium (B).

Fig. 2A is adapted from Poole et al (2018)

Symbiosomes are the cells in which fixation occurs. Within the symbiosome the rhizobia differentiate into bacteroids. This differentiation is driven by plant-nodule-specific cysteine rich peptidases (NCRs) (Montiel et al., 2017). NCRs are transported into the symbiosome where they trigger the differentiation. This differentiation results in the bacteria swelling, undergoing endoreduplication and forming into a Y shape (Fig. 3). There are also large transcriptomic changes within the bacteroid compared to the undifferentiated bacteria as non-essential genes are down-regulated and the expression of the *nif* and *fix* genes begins. This differentiation into endoreduplicated non-replicating bacteroids is an element of this symbiosis found in several clades of legumes and is particularly well studied in members of the Inverted Repeat-Lacking Clade (IRLC), which includes peas (Montiel et al., 2016).

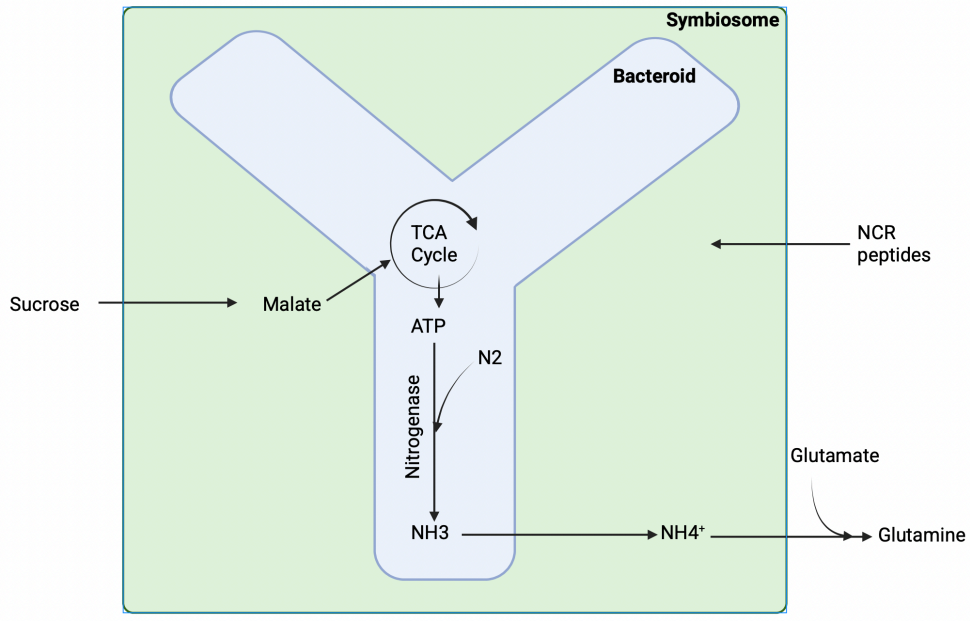

Fig. 3 Symbiosome and bacteroid activity

Nitrogen fixation occurs within specialised plant cells called symbiosomes. Within the symbiosome rhizobia are exposed to NCR peptides which trigger terminal differentiation into Y-shaped bacteroids. Bacteroids are provided with malate by the plant. This feeds into their TCA cycle to provide the energy required to reduce N_2 into ammonia through the activity of the enzyme nitrogenase. Ammonia is exported out of the bacteroid into the symbiosome as ammonium before being transported out of the symbiosome where it is used to convert the amino acid glutamate into glutamine.

A key obstacle in the function of nitrogenase within a nodule is the sensitivity of the enzyme to oxygen. Nitrogenase rapidly breaks down in the presence of oxygen. As such nitrogen-fixation is only triggered in hypoxic conditions. These conditions are created by the plant due to the oxygen impermeable nodule membrane. However, some oxygen is still required for the rhizobia for respiration therefore oxygen is shuttled to the bacteria in small quantities bound to the plant protein leghaemoglobin (Pal et al., 1976).

To ensure nitrogenase is only produced in hypoxic conditions a number of oxygen sensing systems have been developed by rhizobia. In the case of *Rhizobium leguminosarum there* are two oxygen sensing systems. These systems are the FnrN transcription factor and two variants of FixL protein. FixL is a membrane bound protein which under microaerobic conditions phosphorylates FxkR and then FixK to induce downstream *fix* genes. FnrN contains an N-terminal cysteine rich cluster which doesn't bind to DNA in the presence of oxygen. Under microaerobic conditions FnrN binds upstream of the same *fix* genes induced by FixL (Rutten et al., 2021).

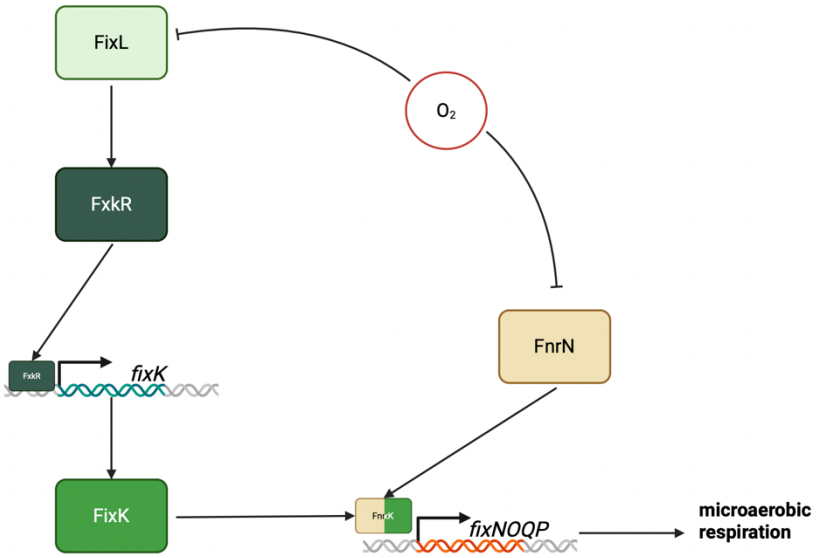

Fig. 4 Oxygen sensing pathways in *R. leguminosarum bv.* 3841

Nitrogen fixation requires hypoxic conditions. To ensure fixation is only triggered under these conditions *R. leguminosarum* has multiple oxygen sensing pathways. In the FixL pathway FixL is only active in the absence of oxygen. FixL phosphorylates FxkR which binds upstream of of *fixK* driving its expression. FixK binds upstream of the *fixNOQP* operon. The FnrN system only requires FnrN which in the absence of oxygen binds to the upstream region of the *fixNOQP* operon. The *fixNOQP* operon is required for the microaerobic respiration which makes powering N_2 fixation under hypoxic conditions possible.

To fuel the fixation of N_2, sucrose is transported from the phloem into the nodule where it is converted into malate by the action of malate dehydrogenases. This malate is transported into the symbiosome where it is taken up by the bacteroid and it feeds directly into the TCA cycle (Schulte et al., 2021). The product of this fixation is ammonia which is then transported out of bacteroid and the symbiosome during which it is converted into ammonium. This ammonium is then used to convert glutamate into glutamine and then into asparagine (Fig. 3). This amino acid is the form in which the fixed nitrogen is then transported out of the nodule for use by the plant (Allaway et al., 2000).

1.3.iv Cheating

Fixing atmospheric N_2 requires a large amount of energy, 16 ATP to form two ammonia molecules (Inomura et al., 2017). Given this large energy cost for fixation there is the potential for a fitness benefit for rhizobia that fix less nitrogen and instead use the energy for replication. This potential benefit should create a selection pressure for bacteria to cheat. This cheating, if unpunished, would result in a 'race to the bottom' as rhizobia fix less and less nitrogen in order to use more energy for replication until eventually the symbiosis no longer provides any benefit to the legume. At this point the relationship may become a parasitism or collapse altogether as the host plant is under a selection pressure to withdraw from the symbiosis (R. F. Denison, 2000).

The apparent stability of the symbiosis, emerging more than 60 million years ago (De La Peña et al., 2018), suggests that there must be a mechanism in place to reduce the fitness of cheating bacteria, thereby maintaining the stability of the symbiosis. The nature of this mechanism was a hotly debated topic for a number of years with several theories put forward. The two main theories for regulation of cheating were partner choice, in which the legume preferentially allows infection by more effective nitrogen-fixing strains, and sanctioning, in which all compatible rhizobia infect, but less effective nitrogen-fixing strains are punished post-infection. Several studies provided evidence supporting sanctioning (Kiers et al., 2003; Oono et al., 2011; S. A. West et al., 2002) before Westhoek et al., (2017) demonstrated that in *Pisum sativum* the host could not distinguish between near isogenic rhizobia, differing only in fixation ability, but could punish those nodules containing a non-fixing strain. Sanctioning results in a reduction

in nodule size and a colour change from pink to white. This colour change is significant as the pink colour of a healthy nodule comes from the leghaemoglobin.

1.3.v Conditional sanctions

This result was taken further by the follow up paper Westhoek et al., (2021). In this study an intermediate strain was used. This strain was able to fix nitrogen at approximately 50% the capacity of a wild-type strain. By comparing the treatment of an intermediate strain when co-inoculated with a non-fixing or a wildtype strain the authors were able to demonstrate that the application of sanctions was conditional on the ability of the strains present. An intermediate strain would therefore be punished when co-inoculated with a wild-type strain but was rewarded when co-inoculated with a non-fixing strain. This result showed that the application of sanctions was not based upon a threshold level of nitrogen output. Instead, the host compares the outputs of nodules and preferentially allocates resources to those of the highest fixation effectiveness.

1.3.vi Mechanism of sanctioning

It has been shown that sanctioning results in the cessation of carbon transport to a sanctioned nodule approximately twenty days post inoculation (Westhoek et al., 2021). This result suggests that one of the mechanisms to punish a less effectively fixing strain is to withhold the carbon supply to the nodule thereby ‘starving out’ the strain. It has also been shown that the metabolic make-up of a sanctioned and unsanctioned nodule is significantly different (Agtuca et al., 2020). Crucially, there is

a drop in the quantity of haem groups in a sanctioned nodule relative to an unsanctioned nodule. From this it may be inferred that within the sanctioned nodules there is no oxygen supply for the rhizobia. This is supported by the change in colour from pink to white seen in a sanctioned nodule (Westhoek et al., 2017). Therefore, it is believed that within a sanctioned nodule the carbon and oxygen supply is stopped thereby punishing the strain within and removing any potential fitness benefit.

This punishment must act on the undifferentiated bacteria to impact the fitness of the strain as only these individuals are able to replicate. Intriguingly when the number of undifferentiated bacteria was measured in a sanctioned nodule 28 dpi there was an increase in the number of viable rhizobia within a sanctioned nodule compared with an unsanctioned nodule. However, by 56 dpi the number of viable rhizobia collapsed in sanctioned nodules (Westhoek et al., 2021). As nodule release does not occur until a much later time point this later collapse would ensure a reduction in fitness for a cheating strain.

1.3.vii Mixed nodules and sanctioning

Mixed nodules occur when multiple strains are captured within the same curled root hair. These strains may then divide down the infection thread where they will then form symbiosomes. However, due to the nature of symbiosome formation, exocytosis from infection droplets, symbiosomes will only ever contain a single strain. Furthermore, each individual plant cell only contains symbiosomes of one strain. Mixed nodules have been shown to occur at high frequency (~20%) in the presence of multiple compatible strains (Mendoza-Suárez et al., 2020). Mixed nodules raise important

evolutionary questions as they may allow a less effectively fixing strain to ‘piggyback’ into a nodule with a more effectively fixing strain and thereby evade sanctioning.

Several studies have investigated how strains are treated within a mixed nodule and whether the host can discern between the two strains and, at the symbiosome level, punish the less effectively fixing strain. However, the studies have produced contradictory results. In Regus et al., (2017) and Daubech et al., (2017) based on confocal and electron microscopy it was proposed that within mixed nodules the symbiosomes containing the less effectively fixing strain underwent premature senescence indicating the host was able to carry out symbiosome level sanctioning. However, in Agtuca et al., (2020) site specific mass spectrometry showed that there was no metabolic difference between the different zones containing the different strains within a mixed nodule despite there being significant metabolic differences in whole nodules containing each of the two strains. As such, it remains an open question as to whether or not legumes can differentiate between and punish accordingly the different strains within a mixed nodule.

1.4 Research Objectives

1. Internal effects of sanctioning

My first objective in this thesis was to understand the effects of sanctioning on the strain within a sanctioned nodule. Importantly, this includes the effect on the bacteroids as well as the undifferentiated bacteria. Parallel to this I aimed to understand the link

between the morphological change to the whole nodule and the effect on the cells within the nodule.

2. Mixed nodules

Given the contradictory evidence available for the treatment of strains within a mixed nodule I studied the treatment of both bacteroids and bacteria within mixed nodules as well as the plant cells containing either strain. By comparing these results to the results from objective one I resolved the question of mixed nodule sanctioning.

3. Avenues to evade sanctions

Given the known diversity in fixation capacity of wild rhizobia strains it was reasonable to assume that there must be some routes for less effectively fixing strains to evade sanctioning. I therefore tested several potential routes for the evasion of sanctioning to determine whether there is a viable option for cheating strains.

4. Regulation of sanctioning

My final objective in this project was to investigate how sanctioning is controlled by the host plant. Particularly, how plants are able to detect and compare nodule outputs before then accurately applying sanctions to the nodules containing less effectively fixing strains.

2 Materials & Methods

Each results chapter contains an individual methods section. This chapter provides media recipes for all of the media used in this thesis as well as a detailed method for the acetylene reduction assay as this is the only method not fully outlined in the methods section of each chapter.

2.1 MEDIA

Growth Media

Bacterial growth

Rhizobium leguminosarum strains were grown on tryptone yeast (TY) (0.3% yeast extract, 0.5% tryptone and $CaCl_2$). When grown on solid media TY agar was made up with 1.75% w/v agar.

Germination plates

Seeds were germinated on 1% w/v water agar plates made up with sterile H_2O.

Rooting solution

Peas were grown in sand and vermiculite with a sterile nitrogen free rooting solution. This solution was made up of:

2.67mM $CaCl_2 \bullet 2H_2O$

267 µM KCl

2.136 mM $MgSO_4 \bullet 7H_2O$

26.7 µM FeEDTA

93.45 µM H_3BO_3

2.136 µM $ZnCl_2$

1.335 µM $Na_2MoO_4 \bullet 2H_2O$

0.801 µM $CuSO_4 \bullet 5H_2O$

9.80 mM KH_2PO_4

10.7 mM Na_2HPO_4

Made up to the required volume using sterile water.

Harvest Solution

Root nodules for flow cytometry were crushed and diluted in Harvest solution (0.9% NaCl, 0.02% SILWET L-77).

Confocal microscopy

Nodules for confocal microscopy were suspended in 8% w/v water agar.

2.2 ACETYLENE REDUCTION ASSAY

Acetylene reduction assays are used to measure the fixation rate of a nitrogen-fixing bacteria due to the carbon-to-carbon triple bond within the molecule's structure which is able to bind to the nitrogenase enzyme as it is structurally similar to the nitrogen

triple bond in N_2. The nitrogenase enzyme then breaks this triple bond leading to the formation of ethylene. The fixation rate may therefore be calculated as a function of the percentage of acetylene converted to ethylene.

For single pot experiments, plants were harvested and the whole plant placed into a 250 ml Schott bottle. For split root assays the two root sections were separated from each other and from the shoot using a sterile scalpel.

Schott bottles were sealed with a lid containing an airtight neoprene seal. Using a syringe, 8 ml of air was removed from each bottle. Acetylene gas was bubbled through water via a rubber hose and 2% of the total bottle volume (6.4 ml) of Acetylene gas was taken by syringe through the rubber hose and injected into the Schott bottle. The difference in volume removed and added to the Schott bottle creates a pressure differential between the bottle contents and the surrounding air. The bottle is then left for one hour. After one hour three 1 ml samples of gas from each Schott bottle were taken. Samples were analysed for acetylene and ethylene concentration through gas chromatography using a Clarus 480, PerkinElmer gas chromatograph.

The fixation rate was calculated in nmoles of ethylene fixed per hour per nodule using the formula given in Fig. 1. The values for the percentage of acetylene and ethylene within each sample were calculated by the gas chromatograph and then averaged across the three samples taken from a single Schott bottle.

$$\frac{\text{Acetylene added (\%)}}{100} \times \frac{\text{Total volume}}{\text{molar volume (cm}^3\text{/M)}} = \text{nmoles of acetylene added}$$

$$\frac{\text{Ethylene (\%)}}{\text{Ethylene (\%) + Acetylene (\%)}} = \text{Fraction converted}$$

$$\frac{\text{Fraction converted x nmoles of acetylene added}}{\text{Number of hours}} = \text{nmoles of ethylene h}^{-1}$$

$$\frac{\text{nmoles of ethylene h}^{-1}}{\text{number of nodules}} = \text{nmoles of ethylene h}^{-1}\ \text{nodule}^{-1}$$

Where:

Acetylene added (%) = 2%
Total volume = 320cm^3
Molar volume = 24.465x10^3 cm^3/M
Ethylene (%) = value from gas chromatography
Acetylene (%) = value from gas chromatography
Number of hours = 1

Fig. 1. Formulae for calculating fixation rate from acetylene reduction assay

The fixation rate for a strain is calculated using nodules occupied by the strain on peas harvested 28 days post inoculation. Plants were placed in Schott bottles with a known volume of acetylene and the fraction of this acetylene converted to ethylene in one hour is calculated by gas chromatography. The total number of nodules on each plant are counted by hand. The final value for fixation rate is calculated as the nmoles of ethylene converted per hour per nodule.

3: internal effects of sanctioning & mixed nodules

3.1: INTRODUCTION

In this chapter, I present my findings in the form of a manuscript that has been submitted for publication, is currently undergoing peer review, and is also available as a pre-print (Underwood et al., 2024). This manuscript is supplemented with a description of work on fluorescence-based carbon transport reporters.

The primary aim of this work was to address key outstanding questions arising from the studies of Westhoek et al. (2017, 2021), which together established the foundational understanding of both sanctioning and conditional sanctioning in this system. Two critical gaps motivated the present study: (i) the intra-nodular consequences of sanctioning on the bacterial and bacteroid populations within the nodules, and (ii) the way in which host plants treat nodules containing multiple strains.

Previous work had demonstrated that sanctioned nodules exhibit a pronounced reduction in size by 28 days post-inoculation (dpi) (Westhoek et al., 2017) and that this reduction is preceded by a marked decrease in carbon supply by 20 dpi (Westhoek et al., 2021). However, intriguingly, the number of viable rhizobia within sanctioned nodules appeared to remain stable, or in some cases even increase, at the same time point when nodule size was already declining. By 56 dpi, immediately prior to nodule senescence and the release of rhizobia into the soil, the population of viable rhizobia collapses, consistent with the long-term effects of sanctioning.

This observation does not explain why nodule size begins to decrease much earlier. One hypothesis is that the reduction in size reflects a decline in the number of bacteroids rather than free-living bacterial cells. Since bacteroids are terminally differentiated and incapable of replication, they would not contribute to measurements of viable rhizobia. This raises a further question: If bacteroid numbers are indeed driving this decrease in size and there is no drop in the number of bacteria by 28 dpi, how can this drop in carbon supply after 20 dpi be reconciled with the unchanged bacterial population? In short, why are the bacterial populations within these nodules apparently unaffected despite over a week of carbon starvation?

The second area addressed was the treatment of mixed nodules. While some studies have argued for the presence of cell-level sanctioning in such nodules (Daubech et al., 2017; Regus et al., 2017), others have presented evidence to the contrary (Agtuca et al., 2020). Notably, two of these studies (Regus et al., 2017 and Agtuca et al., 2020) utilised legume systems that produce determinate nodules and only Daubech et al (2017) uses an indeterminate system. Given the physiological differences between determinate and indeterminate nodules it is entirely possible that the treatment of mixed nodules in the two systems will be different. The work in this chapter on the indeterminate nodules of *Pisum sativum* help to broaden the current understanding of mixed nodules and sanctioning across model systems.

Our approach was to build on the insights gained from our analysis of sanctioned nodules in the first part of this work and apply that framework to assess how mixed nodules are treated by the host.

Understanding how host-plants respond to a mixed nodule is crucial in order to properly understand the effect of sanctioning on a heterogenous population of rhizobia as is found in the rhizosphere. Rhizobia present in 'wild' populations vary significantly in their ability to fix-nitrogen (Berrada et al., 2019; Woliy et al., 2019), therefore, the question must be asked as to how these less effective fixers have persisted in the face of sanctioning. If mixed nodules are subject to cell-level sanctioning, it would imply that inefficient strains cannot escape the fitness costs of sanctions by co-inhabiting nodules with more effective partners. Conversely, if sanctioning occurs only at the whole-nodule level, it suggests that mixed occupancy might provide a partial refuge for less effective fixers, with important consequences for the maintenance of diversity in the rhizosphere.

By investigating these aspects of sanctioning, the aim is not only to answer these specific questions but also to develop a deeper understanding of the underlying mechanism by which sanctions are imposed. A central theme throughout this project, and particularly in the first two results chapters of this thesis, was to use experimental data to infer the characteristics of the sanctioning mechanism with a view to generating hypotheses about its genetic and physiological basis. This broader understanding ultimately informed the approach taken in the final results chapter, where the objective was to probe the mechanism more directly.

3.2: PEA PLANTS CONDITIONALLY SANCTION LESS EFFECTIVELY FIXING RHIZOBIA AT THE LEVEL OF WHOLE NODULES RATHER THAN SINGLE CELLS

3.2.i Forward

This section of the thesis contains the manuscript 'Pea plants conditionally sanction less effectively fixing rhizobia at the level of whole nodules rather than single cells' as submitted for peer review and available for pre-print (Underwood et al., 2024). Minor formatting changes have been made to this manuscript to fit the format of this thesis. The supporting information for this chapter is provided in Appendix A. The reference list has been moved and combined with the overall thesis bibliography.

3.2.ii list of supporting information

The following Supporting Information is available for this article:

Fig. S1 Image of flow cytometry output showing bacteroid and undifferentiated bacterial populations differentiated by size based on Forward Scatter (FSC)

Doc. S2 Flow cytometry data analysed using custom gating to calculate the number of undifferentiated bacteria and bacteroids in single occupancy and mixed nodules, and acetylene reduction assay and nodulation results from fluorescent tag control experiments.

Doc.S3 R markdown file of statistical analysis.

Fig. S4 Formulae for back transformation for outputs of analyses of $\log_{10}$ transformed

data

Fig. S5 Representative iBright image of pea inoculated with two strains of differing effectiveness.

Fig. S6 Representative confocal images of nodule sections of both nodule types from all three co-inoculation combinations (Fix^+ vs Fix^-, Fix^+ vs Fix^{int}, Fix^{int} vs Fix^-) at 28-, 35- and 42-days post inoculation.

Fig. S7 Analysis of the number of bacteria of each strain within a mixed nodule

Fig. S8 Analysis of the number of bacteria within a mixed nodule compared to the nodule controls

3.2.iii Abstract

Legumes sanction root nodules containing rhizobial strains with low nitrogen fixation rates (less effectively fixing). Pea (*Pisum sativum*) nodules contain both undifferentiated bacteria and terminally differentiated nitrogen-fixing bacteroids. It is critical to understand how sanctions act on both bacteria and bacteroids, and how they differ. In addition, less effective strains could potentially evade sanctioning by entering the same nodule as an effective strain i.e., piggybacking. *P. sativum* was co-inoculated with pairwise combinations of three strains of rhizobia with different effectiveness, to test whether ineffective strains can evade sanctions in this way. We assessed the effect of sanctions on nodule populations of bacteria and bacteroids using flow cytometry and the effects on nodule internal structure using confocal microscopy. We

show that sanctioning lowered bacteroid populations and caused a reduction in the size of bacteria. Sanctions also precipitated an early change in nodule cell morphology. In nodules containing two strains that differed in their nitrogen-fixation ability, both were sanctioned equally. Thus, peas sanction whole nodules based on their nitrogen output, but do not sanction at the cellular level. Our results demonstrate peas conditionally sanction at the whole nodule level, providing stability to the symbiosis by reducing the fitness of ineffective strains.

3.2.iv Introduction

Legumes have overcome nitrogen limitation by establishing a mutually beneficial interaction with nitrogen-fixing bacteria, hosted within nodules along their root systems (Poole et al., 2018). Certain species of legumes, like peas, form nodules in which some bacteria undergo terminal differentiation to become large swollen cells called bacteroids, which carry out the costly process of nitrogen fixation, but cannot resume their free-living existence (Mergaert et al., 2006). The plant provides carbon compounds in the form of photosynthetically-derived dicarboxylates in return for ammonia provided by the bacteroids, and this relationship continues until the plant's nitrogen requirements have been met (Udvardi & Poole, 2013). At this point nodule senescence ensues, and while the bacteroids then die, any undifferentiated bacteria are released into the soil (Timmers et al., 2000). The presence of a host legume leads to an increase in the soil population of rhizobia (Beringer et al., 1979); therefore, by engaging in the symbiosis, legumes potentially provide a significant fitness advantage to rhizobia.

However, this interaction presents an evolutionary dilemma. A ‘cheating’ strain that reduces investment in the costly process of nitrogen fixation, might be able to produce more reproductive bacteria within the nodules, potentially giving it a fitness advantage. Legumes are known to have evolved sanctions to punish cheating bacteria (Kiers et al., 2003; Oono et al., 2011; S. A. West et al., 2002; Westhoek et al., 2017) and these sanctions can be applied conditionally, with pea plants known to sanction nodules that contain an intermediate-fixing strain, only when a better strain is available (Westhoek et al., 2021). Intriguingly, while Westhoek et al (2021) did see a significant drop in carbon transport and nodule size by twenty-eight days post inoculation (dpi), they did not see a significant decrease in the number of viable bacteria within the sanctioned nodule (although the number of viable bacteria had plummeted dramatically by 56 dpi). As such, the cause of the reduction in nodule size at 28 dpi, while hypothesised to be due to a drop in the bacteroid population, has yet to be proven.

Poorly fixing strains might be able to evade plant sanctions by ‘piggybacking’ on a more effective strain, since multiple strains can occupy a single nodule at frequencies of over 20% (Mendoza-Suárez et al., 2020). Notably individual cells of ‘mixed’ nodules only contain one bacterial strain. If plants distinguished between cells containing different strains they could prevent piggybacking by sanctioning cells containing the less effective strain. Despite some evidence for so-called cell-autonomous sanctioning, in the form of premature senescence of cells containing an ineffective strain (Daubech et al., 2017; Regus et al., 2014), metabolic evidence has suggested that this does not occur (Agtuca et al., 2020), so it remains contentious.

This study builds on previous work carried out by Westhoek et al. (2021) using pea plants and a set of otherwise isogenic rhizobia strains that differ in their ability to fix nitrogen. First, with nodules infected with a single rhizobial strain, we used flow cytometry to measure fitness characteristics, of both undifferentiated bacteria and nitrogen-fixing bacteroids, we then used fluorescence microscopy to establish how sanctions influenced the health and morphology of the infected plant cells within those nodules. Second, we used the same techniques on mixed nodules to determine whether or not pea plants carry out cell-autonomous sanctioning within nodules. Our results show that sanctions reduce the number of bacteroids within whole nodules as well as reducing the size of the undifferentiated bacteria. Fluorescence microscopy showed that the breakdown of cells within the nodule occurred prematurely in sanctioned nodules. Our results also show that these sanctions operate at the level of the whole nodule but not at the cellular level. As such, a piggybacking strain cannot be punished independently of the non-cheater within a mixed nodule; however, mixed nodules were treated as ineffective and sanctioned at the nodule level. As such, piggybacking is not an effective route by which cheaters can succeed.

3.2.v Methods

Bacterial Strains and Culture Conditions

Rhizobial strains used in this study are all derivatives of a highly effective nitrogen-fixing strain, *Rhizobium leguminosarum* bv. (Rlv) 3841, that infects pea (*Pisum sativum* L. cv. Avola) (Johnston & Beringer, 1975). The mutant strains thus differ in their nitrogen-fixation ability but are otherwise genetically identical (Table 1). They are labelled with a fluorescent marker (mCherry or GFP) in order to distinguish the nodules formed by each strain (Table 1). Strains were maintained on tryptone-yeast (TY) agar (Beringer, 1974) with the appropriate concentrations of antibiotics (Table 1). For long-term storage, strains were kept at −80 °C in TY with 15 to 20% glycerol. Rhizobial inoculant was grown on a TY agar slope and the number of bacteria on the slope was determined by measuring the OD600 of the washed slope using a Genesys 150 UV-Visible spectrophotometer. Cells were diluted to approximately 5 $x10^7$ ml^{-1}.

Plant Growth

Before sowing, all pea seeds were surface sterilized (1 minute in 95% ethanol followed by 5 minutes in 20% NaClO), rinsed, and left to germinate on 1% w/v agar plates at room temperature in the dark. Seedlings were transplanted after five days by transferring them to sterilized 1 litre Azlon beakers containing a 1:1 mixture of silver sand and fine vermiculite, 150 ml sterilized nitrogen-free nutrient solution, and a 1:1 ratio of the two rhizobial strains (approximately 0.25 x 10^7 cells) (see also Westhoek et al., (2017)). Beakers were covered with clingfilm to reduce aerial contamination,

which was slit after a few days to allow seedlings to grow through. Plants were grown in a growth room (21 °C, 16 h photoperiod) for 28 to 42 days and watered as necessary from 7 days onwards.

Bacterial inoculation of Plants

Plants were inoculated with either single strains or three pairs of otherwise isogenic strains, differing only in their ability to fix nitrogen. The strains are: *wildtype* or *good fixer* (Fix^+); *intermediate fixer* (Fix^{int}) and *non-fixer* (Fix^-). This gives three pairwise combinations of a more effective (E) and a less effective (L) strains; Fix^+ (E) vs Fix^- (L), Fix^+ (E) vs Fix^{int} (L) and Fix^{int} (E) vs Fix^- (L). i.e., Fix^+ is always effective and Fix^- always less effective, but Fix^{int} can be either effective or less effective, depending on the co-inoculated strain. In each case the more effective strain was tagged with mCherry and the less effective strain with GFP. Two sets of plants, both containing six replicates of each pairwise combination, were initially set up for the collection of nodules for flow cytometry. Additional plants were set up in sets of five replicates per pairwise combination for confocal imaging of nodules at various time points.

Table 1: Rhizobial strains and their fixation abilities.

All strains derived from Rlv3841 and provided with a strain code, resistance markers, short description and reference.

Name	Strain	Antibiotic resistance	Description	Reference
Fix^{+}	Rlv3841	Streptomycin	Rlv3841	(Johnston & Beringer, 1975)
Fix^{+} mCherry	OPS1341	Streptomycin, Gentamicin	Rlv3841 Tn*7*-Gm-mCherry	(Westhoek et al., 2021)
Fix^{+} GFP	OPS1339	Streptomycin, Gentamicin	Rlv3841 Tn7-Gm-GFP	(Westhoek et al., 2021)
Fix^{-} GFP	OPS2270	Streptomycin, Spectinomycin, Gentamicin	Ω*nifH* Tn*7*-Gm-sfGFP (Rlv3841 mutant, ΩSpc cassette in *nifH*)	(Westhoek et al., 2021)
Fix^{int} GFP	OPS2268	Streptomycin, Spectinomycin, Gentamicin	Fix^{int} Tn*7*-Gm-sfGFP (Rlv3841 mutant, ΩSpc cassette in promoter region of *nifA*)	(Westhoek et al., 2021)
Fix^{int} mCherry	OPS2269	Streptomycin, Spectinomycin, Gentamicin	Fix^{int} Tn*7*-Gm-mCherry False colour: orange	(Westhoek et al., 2021)

Harvesting

For flow cytometry, plants were harvested at 28 dpi, for confocal microscopy they were harvested at 28-, 35- and 42- dpi. Plants were harvested by removing them from the sand/vermiculite mixture and washing the roots carefully. Nodules were then imaged using a LEICA M165 FC fluorescent stereo microscope and an iBright FL1500 imaging system. Nodule occupants were identified based on their fluorescence. When selecting single occupancy nodules, the five largest of each nodule type were selected. When selecting mixed nodules they were not selected based on size. Instead all mixed nodules identifiable on the plant were selected due to limited numbers of mixed nodules.

Confocal imaging

To assess the health of cells containing undifferentiated bacteria and bacteroids within nodules, sections were imaged using confocal microscopy. This allows the visualisation of fluorescently tagged strains and the discrimination of the two strains within a mixed nodule. Nodules were placed in 8 % w/v agar and 100 µm longitudinal sections were cut through the centre of the nodule using a Leica VT1200S vibratome. Nodule slices were then imaged with a Zeiss LSM 880 Airy Scan confocal microscope and analysed with ZEN Black software. To visualise fluorescent tags, mCherry was excited using a 561 nm wavelength laser and emissions detected between 598 and 649 nm, while GFP was excited using a 488 nm wavelength laser and emissions detected between 498 and 562 nm.

Flow Cytometry

To identify and quantify the population sizes of undifferentiated bacteria and bacteroids within nodules, flow cytometry was used. This was applied to single occupant nodules from the three co-inoculant combinations (Fix^+ vs Fix^-, Fix^+ vs Fix^{int} and Fix^{int} vs Fix^-) and to mixed nodules containing Fix^+ & Fix^-. All nodules were prepared by placing them in a 1.5ml Eppendorf tube with 300µl of harvest solution (0.9% NaCl, 0.02% SILWET L-77) and crushing them with an autoclaved microcentrifuge pestle. This solution was passed through a 40µm filter and diluted tenfold to increase the accuracy of the flow cytometry.

Flow cytometry was conducted with an Amnis® CellStream® flow cytometer (Luminex) equipped with a 488 and 561nm lasers, which were used for excitation of GFP and mCherry, respectively. Flow rates were set to low speed and high sensitivity (3.66 µL/min) and the Flow cytometer was set to run 10 µl per sample. Analysis of flow cytometry data was carried out using the CellStream® Analysis software (Version 1.5.17). Bacterial events were defined based on custom gating parameters. Singlets and doublets were gated with a threshold of 0.4 forward scatter (FSC) aspect ratio. Bacterial singlets which emitted at 611/631nm, when excited at 561 nm with an intensity above 6,000 arbitrary units (AU) were defined as red. Bacterial singlets with emission at 528/546nm, when excited at 488nm, with an intensity above 4,000 AU were defined as green.

Bacteroids and bacteria were defined based on size, as in Mergaert et al., (2006), using custom gating of the FSC detection to separate the two populations (Figure S1). The flow cytometer calculates a value of events per ml from the number of counts within the 10 µl. The flow cytometer assumes the sample comes from a volume of 1ml (rather than the 300 µl we had per sample). As the samples underwent a ten-fold dilution, in order to calculate the true events per nodule we multiplied the flow cytometer calculated value by three. Flow cytometry data available at http://zenodo.org for the single occupancy experiments separated by inoculum pairing and mixed nodule experiments. Links to data are provided in the data availability section.

Acetylene reduction assay

Fixation rates were measured via an acetylene reduction assay using the method described in Westhoek et al (2021).

Statistical Analysis

To test the effect of the fluorescent markers on the symbiotic characteristics of Rlv3841, we compared the fixation rates of untagged Fix^+, Fix^+ GFP and Fix^+ mCherry using one-way ANOVA. We compared the nodulation competitiveness of the same three strains in pairwise combinations using paired t-tests.

To test for conditional sanctioning at the whole-nodule level, we subsetted the data into the three different nodule types: Fix^+, Fix^- and Fix^{int}, and tested whether three

relevant measures of bacterial fitness and plant sanctioning behaviour were dependent on the identity of the co-inoculated strain. The chosen measures were: (1) the size of the undifferentiated bacterial population, (2) the size of the bacteroid population and (3) the size of individual undifferentiated. We did not measure the size of individual bacteroids because the numbers of bacteroids within some sanctioned nodules was so small as to make this measure unreliable. Both population sizes and sizes of individuals were analysed using mixed-effects models in which individual plant ID was the random effect and the identity of the co-inoculant was the fixed effect.

To test for the presence of cell autonomous sanctioning within mixed nodules, we compared the number of bacteroids, number of bacteria and the mean size of bacteria of the two strains within mixed nodules using a linear mixed-effects model, the random effect was nodule ID nested within plant ID.

To get a better sense of how the plant treats mixed nodules, we compared the total number of bacteroids, total number of bacteria and average size of bacteria in Fix^+ & Fix^- mixed nodules with Fix^+ and Fix^- single-occupant nodules. plant ID was the random effect.

All statistical tests were carried out using R version 4.2.1 (2022-06-23) and R studio 2022.07.2 and graphs were produced using Graph Pad version 9. An R markdown document is provided (Doc. S3). For simplicity, in the figures, numbers of bacteria and bacteroids are always presented $\log_{10}$ transformed, although the log transformation was only required for a subset of analyses in order to meet the

assumptions of equal variance and normality, which was assessed by visual inspection of residual plots (see R markdown file (Doc. S3)). Where $\log_{10}$ transformation was carried out for analysis the statistical outputs have been back transformed for readability. Back transformation carried out using the formulae given in Fig. S4.

3.2.vi Results

Fluorescently tagging strains does not affect symbiotic characteristics of Rlv3841

We first tested whether fluorescent marking alters the competitiveness of rhizobial strains. There was no significant difference in the number of nodules occupied by each strain on plants inoculated with equal numbers of untagged Rlv3841, versus its mCherry and Gfp-tagged versions (Fix^+ vs Fix^+ mCherry: estimate difference in number of nodules = 22.750, SE = 13.288, t = 1.712, $p > 0.05$, df = 6; Fix^+ vs Fix^+ GFP: estimate difference in number of nodules = -5.75, SE = 20.84, t = -0.276, $p>0.05$, df =6). Similarly, plants co-inoculated with mCherry- and Gfp-tagged Rlv3841 did not have significant differences in nodules occupied by each strain (Fix^+ GFP vs Fix^+ mCherry: estimate = -12.250, SE = 11.265, t = -1.087, $p>0.05$, df = 6). In addition, peas inoculated singly with the three strains did not differ significantly in fixation rates per nodule ($F_{2, 9} = 1.387$, $p > 0.05$, n = 4, df = 2). In summary there was no significant difference in any of the measured characteristics between the tagged and untagged strains.

Conditional sanctioning reduces the number of bacteroids and the size of bacteria

Nodules occupied by the intermediate-fixing strain contained significantly fewer bacteroids when co-inoculated with Fix^+ rather than Fix^- bacteria. Thus conditionally sanctioned nodules have reduced bacteroid numbers. As expected, the number of

bacteroids in Fix^+ and Fix^- nodules did not significantly vary depending on the co-inoculated strain (Figure 1A) (Table 2). This is because Fix^+ nodules are never sanctioned and Fix^- nodules are always sanctioned.

Table 2: Impact on number of bacteroids of focal strain with different co-inoculants

The effect of co-inoculant on the number of bacteroids in a nodule. mixed-effects models were used to compare the number of bacteroids within a nodule when co-inoculated with different strains. Data that has been log_{10} transformed is indicated and presented back transformed. P-values exceeding 0.05 are reported as >0.05. The value for difference in bacteroid number is given for the more vs less effective co-inoculant. The t and p value are taken from the mixed effects model and n is the number of nodules.

Strain (co-inoculant comparisons)	Difference in bacteroid numbers due to co-inoculant	Standard error	t	p	n
Fix^+ (Fix^{int} & Fix^-)	970769	2039541	0.476	>0.05	60
Fix^{int} (Fix^+ & Fix^-)	$-4.29x10^6$ (log_{10})	$2.02x10^5$ (log_{10})	6.818	$7.74x10^{-5}$	55
Fix^- (Fix^+ & Fix^{int})	44647(log_{10})	21731(log_{10})	-1.806	>0.05	55

Undifferentiated bacteria from Fix^{int} nodules were significantly smaller when co-inoculated with a Fix^+ rather than a Fix^- strain. Thus, conditional sanctioning reduces the size of undifferentiated bacteria within nodules. As expected, undifferentiated

bacteria from Fix^+ nodules were not significantly altered in size when co-inoculated with Fix^{int} or Fix^- strains. Surprisingly, undifferentiated bacteria from Fix^- nodules were significantly smaller when co-inoculated with a Fix^+ strain, but not when co-inoculated with a Fix^{int} strain (Figure 1B) (Table 3).

Table 3: Impact on size of undifferentiated focal bacterial size of strains with different co-inoculants

The effect of co-inoculant on the size of undifferentiated bacteria in a nodule. mixed-effects models were used to compare the size of bacteria within a nodule when co-inoculated with different strains. Data that has been log_{10} transformed is indicated and presented back transformed. P-values exceeding 0.05 are reported as >0.05. The value for difference in size is given for the more vs less effective co-inoculant. The t and p value are taken from the mixed effects model and n is the number of nodules.

Strain (co-inoculant comparisons)	Difference in bacterial size due to co-inoculant	Standard error	t	p	n
Fix^+ (Fix^{int} & Fix^-)	561.3	301.6	1.861	0.0923	60
Fix^{int} (Fix^+ & Fix^-)	-1417.6	405.9	-3.492	0.0068	55
Fix^- (Fix^+ & Fix^{int})	-450 (log_{10})	170 (log_{10})	-2.566	0.03	55

Finally, the number of undifferentiated bacteria within a nodule did not depend on the identity of the co-inoculant for any of our nodule types (Fix^+ nodules, Fix^- nodules or Fix^{int} nodules). This is consistent with previous studies which found no significant

change in CFU due to conditional sanctioning at 28 dpi (although it does at 56 days (Westhoek et al., (2021)). Overall, conditional sanctioning therefore reduces both bacteroid number and the size of undifferentiated bacteria but not their numbers at 28 dpi. (Fig. 1C) (Table 4).

Table 4: Impact on number of undifferentiated bacteria of each strain with different co-inoculants

The effect of co-inoculant on the number of undifferentiated bacteria in a nodule. mixed-effects models were used to compare the number of bacteria within a nodule when co-inoculated with different strains. Data that has been log_{10} transformed is indicated and presented back transformed. P-values exceeding 0.05 are reported as >0.05. The value for difference in number of bacteria is given for the more vs less effective co-inoculant. The t and p value are taken from the mixed effects model and n is the number of nodules.

Strain (co-inoculant comparisons)	Change to number of bacteria due to co-inoculant	Standard error	t	p	n
Fix^+ (Fix^{int} & Fix^-)	-212437	472483	-0.450	>0.05	60
Fix^{int} (Fix^+ & Fix^-)	$-5.81x10^5$ (log_{10})	$1.17x10^5$ (log_{10})	0.855	>0.05	55
Fix^- (Fix^+ & Fix^{int})	$2.79x10^6$	$2.01x10^6$	1.391	>0.05	55

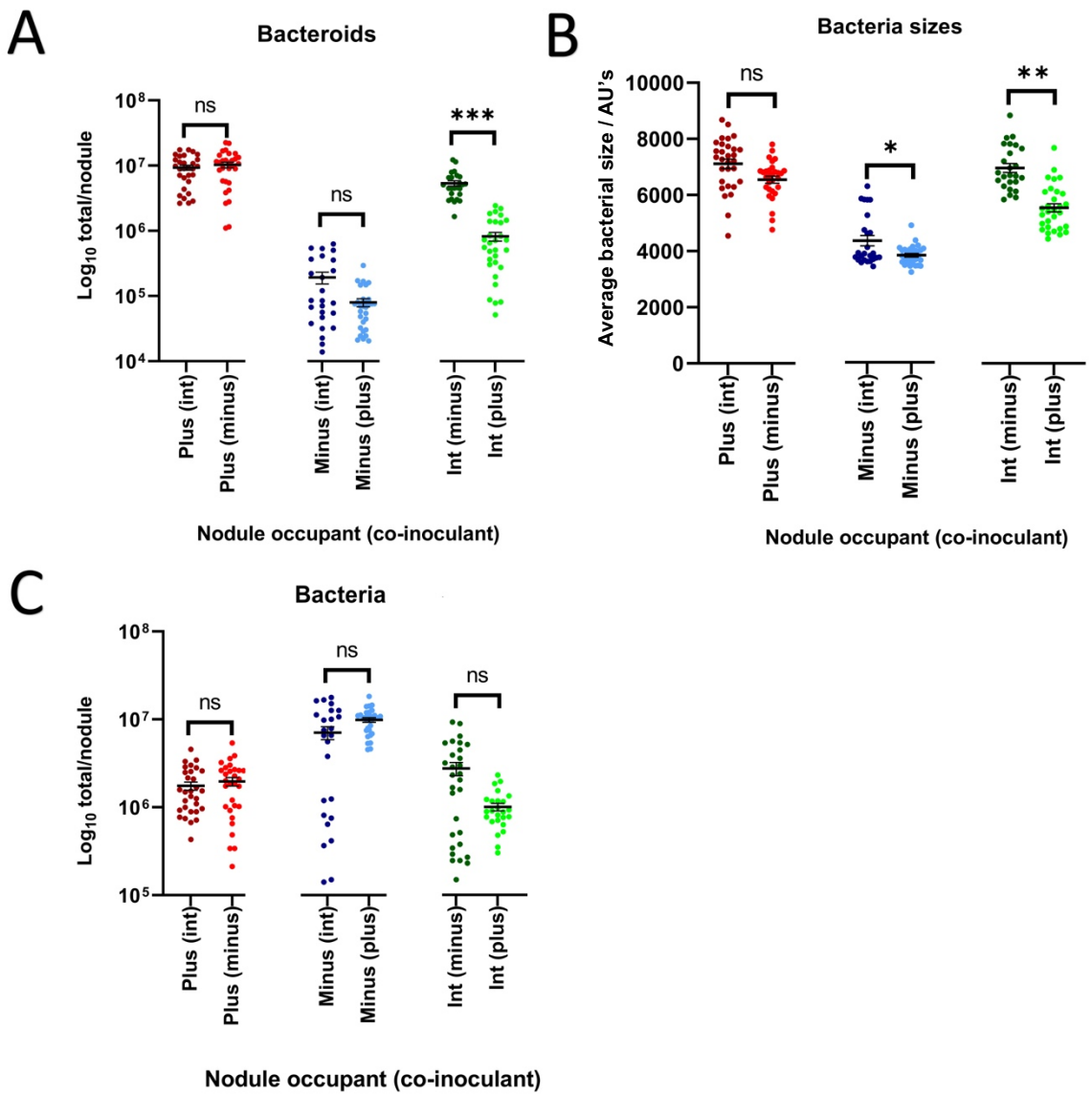

Fig 1. After 28 days, plant-imposed sanctions reduce the number of bacteroids and bacterial size, but not the number of bacteria.

The number of bacteroids (A) the size of bacteria (B) and the number of bacteria (C) in nodules from pea plants co-inoculated with two strains of rhizobia. Data is split into different single-strain nodule types (Fix^{+}, Fix^{int} or Fix^{-}). The identity of the co-inoculant is given in brackets. Horizontal bars give the mean value with one standard error. Significance level from paired t-test *** < 0.001; ** < 0.01; * <0 .05; ns, not significant. All nodules harvested at 28 days post-inoculation. Strains were tagged with a fluorescent protein. Fix^{+} was always tagged with mCherry, Fix^{-} was always tagged with GFP, and Fix^{int} was tagged with mCherry when co-inoculated with Fix^{-} and GFP when co-inoculated with Fix^{+}.

Conditional sanctioning changes nodule cell morphology

After 28 dpi, the size of sanctioned whole nodules is clearly reduced (Figure S4), as also seen in Westhoek et al., (2017). However, to study the effects on cellular morphology of sanctioned nodules, plants were co-inoculated with all three combinations of bacterial strains (Fix^+ vs Fix^-, Fix^+ vs Fix^{int}, Fix^{int} vs Fix^-) and nodules were imaged at 28-, 35- and 42-dpi (Figure S5 for all pictures). At 28 dpi, all sections taken from nodules containing the Fix^{int} strain looked similar, regardless of whether the co-inoculated strain was Fix^- (Figure 2A) or Fix^+ (Figure 2B). However, at 35 dpi, nodules containing the Fix^{int} strain were visibly affected when the co-inoculated strain was Fix^+. Infected cells within Fix^{int} nodules were irregularly shaped and the infected region had retracted from the nodule edge (Figure 2D). By comparison when the co-inoculated strain was Fix^-, most cells retained a round morphology; and were not visibly different from Fix^{int} nodules at 28 dpi (compare panel C with A&B in Figure 2). At 42 dpi, all Fix^{int} nodules showed clear changes to cell morphology, which were most pronounced when co-inoculated with Fix^+ (Figure 2F), rather than Fix^- (Figure 2E). Therefore, conditional sanctioning induces a premature change to the cellular morphology of nodules.

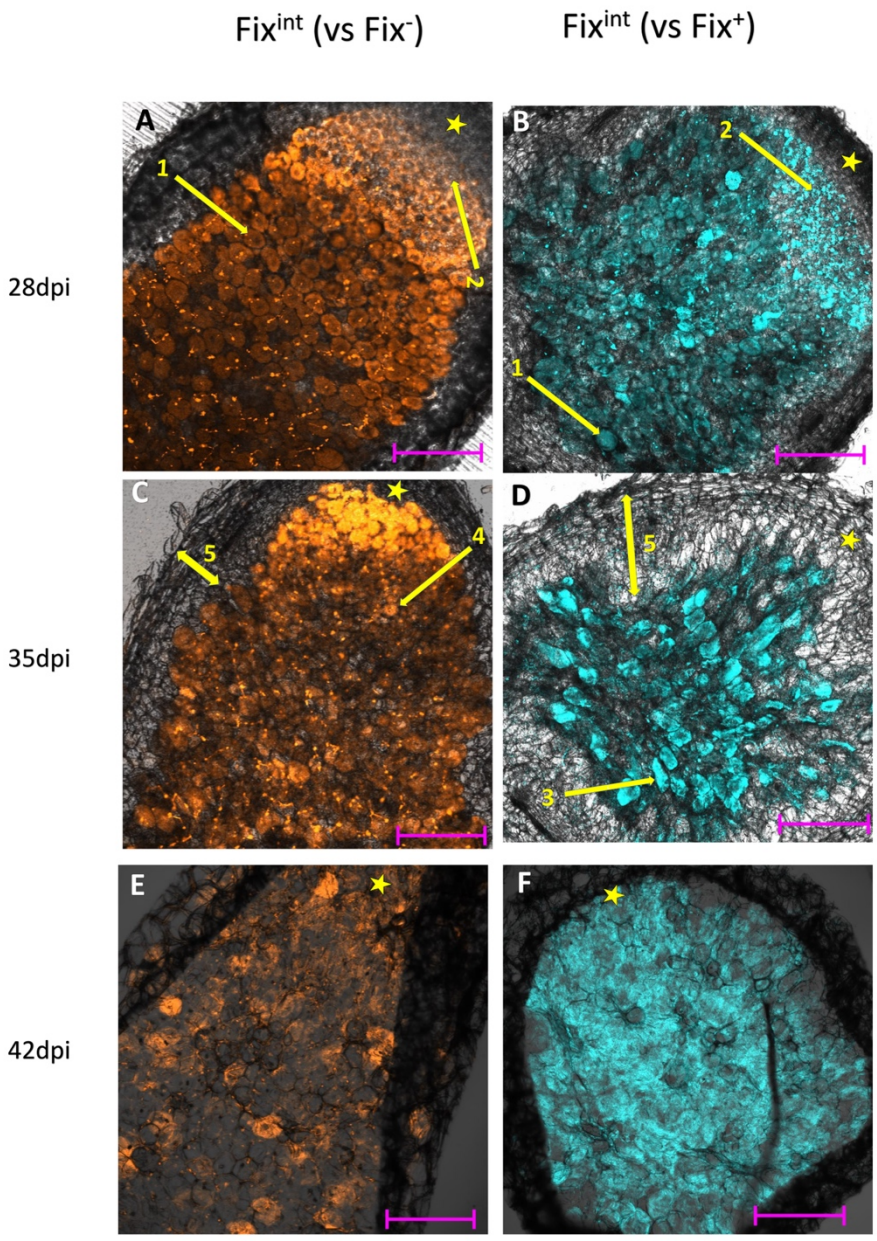

Fig. 2. Fix^{int} nodules co-inoculated with Fix^{+} strain change cell morphology earlier than those co-inoculated with Fix^{-} strain

(A), (C) and (E) Fix^{int} nodule labelled with mCherry (orange), co-inoculated with Fix^{-}. (B), (D) and (F) Fix^{int} nodule labelled with GFP (blue), co-inoculated with Fix^{+}. Nodules were harvested at 28 dpi, 35 dpi and 42 dpi, respectively. Longitudinal nodule slices (100µm) were imaged by confocal microscopy. Tip of the nodule is indicated by a star. Twenty-eight days post-inoculation, regardless of co-inoculant, infected cells were spherical (1) and an actively dividing meristem is visible (2). Thirty-five days post-inoculation, the infected cells in sanctioned Fix^{int} nodules were no longer spherical (3), in contrast to those seen in the unsanctioned Fix^{int} nodules (4). In addition, the area of infected cells in sanctioned nodules shows clear withdrawal from the edge of the nodule compared with unsanctioned nodules (5). Forty-two days post-inoculation there were very few remaining spherical cells in either sanctioned or unsanctioned nodules.

Scale bar = 200µm. Nodule tip is denoted by star.

Conditional sanctioning is not cell-autonomous in pea

Cell-autonomous sanctioning predicts that in nodules containing more than one strain (mixed nodules) the plant will differentiate between the strains and sanction cells containing the less effective strain. This would result in fewer bacteroids and smaller undifferentiated bacteria of the less effective relative to the more effective strain. We tested this theory in Fix^+ and Fix^- mixed nodules as this is the most extreme difference in fixation rates between strains available

When comparing the number of bacteroids of each of the two strains within mixed nodules there was no significant difference between the number of Fix^+ and Fix^- (Log_{10} transformed: Estimate = $1.133x10^5$, SE = 73528, t = 1.455, p >0.05, n = 21)(Fig. 3A). This data is consistent with peas being unable to differentiate between the two strains within a mixed nodule.

There was no significant difference in the size of bacteria within mixed nodules between the more effective and the less effective strain (Estimate = 511, SE = 267, t = 1.916, p > 0.05, n = 21))(Fig. 3B). This is also consistent with an absence of cell autonomous sanctioning, as sanctions had a clear impact on the size of undifferentiated bacteria in single occupant nodules (Fig 1B) that is not present in mixed nodules.

As there was no clear impact of conditional sanctioning on the number of bacteria within single occupancy nodules this comparison for mixed nodules has not been considered (Fig. S6).

Based on these results, the evidence does not support cell-autonomous sanctioning in peas. Since this makes piggybacking in mixed nodules a potential route to success for a strain less effective at fixing N_2, we also examined how mixed nodules were treated at the whole nodule level by the plant.

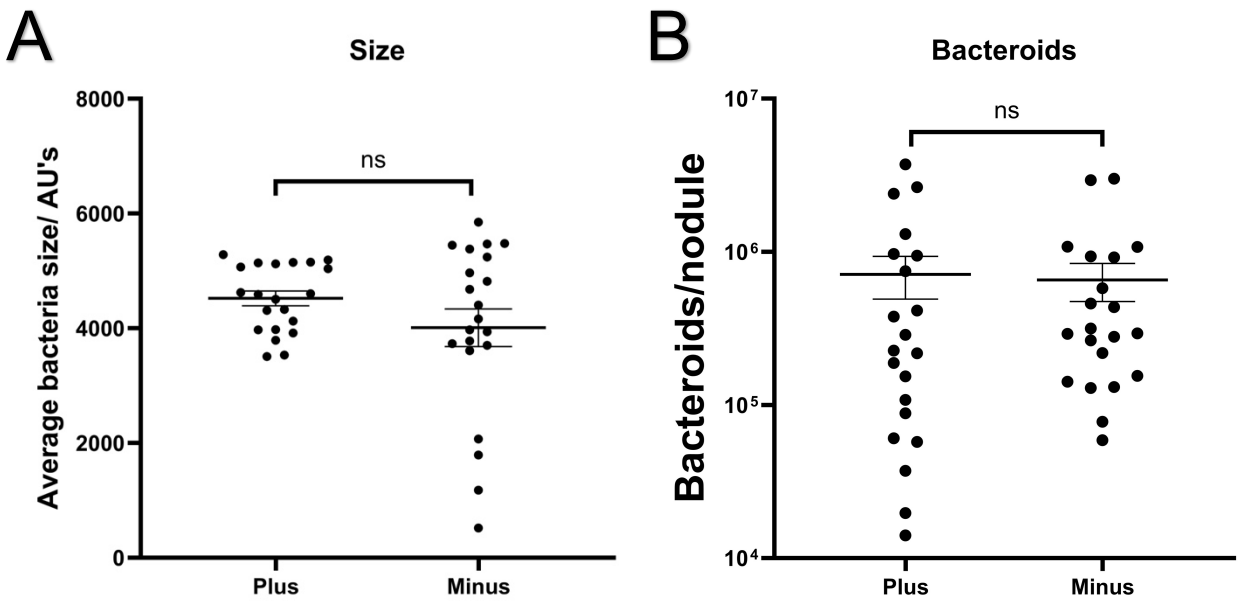

Fig. 3. The two strains within a mixed nodule are not sanctioned independently

The number of bacteroids (A) and the size of bacteria (B) for the two strains within mixed nodules. mixed nodules contained a Fix^+ and a Fix^- strain. Horizontal bars give the mean value with one standard error. Significance level from paired t-test: * <0 .05; ns, not significant. All nodules harvested at 28 days post-inoculation. Strains were tagged with a fluorescent protein. Fix^+ was tagged with mCherry, Fix^- was tagged with GFP.

Mixed nodules are sanctioned

If mixed nodules are sanctioned at the whole-nodule level, then mixed nodules should be sanctioned in the same manner as a single strain intermediate fixing nodule. This is because the total amount of nitrogen fixed by a mixed nodule will be the average of the two strains (and hence similar to an intermediate fixing strain). As seen in Figure 1 when a Fix^{int} nodule was sanctioned it had a significantly reduced number of bacteroids and smaller bacteria. To test this, the total number of bacteroids, bacterial size and bacterial numbers in mixed nodules were compared with the two single occupant nodules taken from plants co-inoculated with Fix^{+} and Fix^{-}. Intermediate sanctioning of mixed nodules predicts that mixed nodules will contain more bacteroids and bigger bacteria than the less effective Fix^{-} nodule while having fewer bacteroids and smaller bacteria than the more effective single Fix^{+} nodule. As was seen for sanctioned Fix^{int} nodules (see Fig. 1).

As predicted, the number of bacteroids within a mixed nodule was significantly lower than the number within the Fix^{+} only nodule (Log_{10} transformed: Estimate = -7.17×10^{6}, SE = 3.02×10^{6}, t = 7.232, $p < 0.001$) (Fig. 4A). The mixed nodules also contained significantly more bacteroids than the within Fix^{-} only nodules (Log_{10} transformed: Estimate = 7.08×10^{5}, SE = 22737, t = 7.914, $p < 0.001$) (Fig. 4A). These results are consistent with mixed nodules being treated at the whole nodule level and sanctioned to the same degree as an intermediate fixing strain.

As predicted, bacteria within mixed nodules were significantly smaller than those within the more effective Fix^{+} nodules (Estimate = -2814, SE = 296, t = 9.513, p

<0.001) (Fig. 4B) This dramatic decrease in size is consistent with mixed nodules being intermediately sanctioned. However, there was no significant difference in size compared to the less effective nodule control (Estimate = 475, SE = 296, t = 1.608, p >0.05)(Fig. 4B). This suggests that the change in size of bacteria is only dramatic when comparing a sanctioned nodule relative to the unsanctioned Fix^+ nodule. In contrast there is not a dramatic change in size when comparing two different types sanctioned nodules e.g. a Fix^- nodule or a mixed nodule of Fix^+ & Fix^-.

As before, the analysis of the number of bacteria within mixed nodules has not been included but statistical analysis and data visualisation was carried out (Fig. S7).

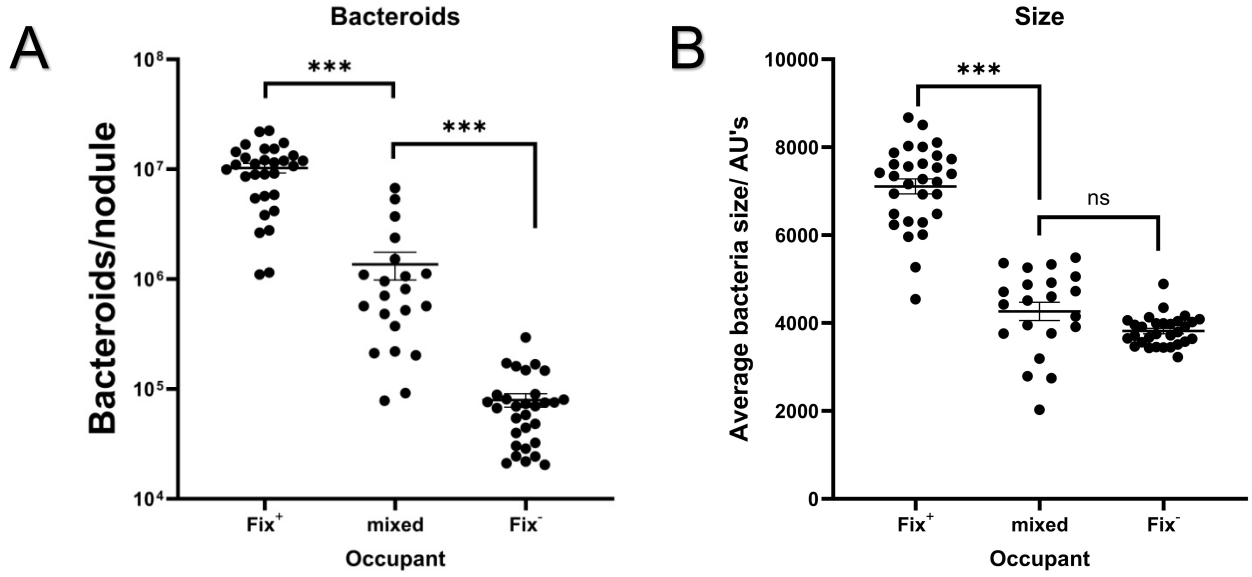

Fig. 4. Mixed nodules are sanctioned, but less severely than single strain nodules containing the less effective strain.

The number of bacteroids (A) and the size of undifferentiated bacteria (B) within pea nodules. Mixed nodules containing Fix and Fix is compared with single occupant Fix and Fix. Bars indicate the mean value and error bars are one standard error. All nodules were harvested 28 days post inoculation. Significance levels from t-test: *** < 0.001; ns, not significant. Strains were tagged with a fluorescent protein. Fix^+ was tagged with mCherry, Fix^- was tagged with GFP.

Changes to cell morphology are not cell-autonomous in pea

Within mixed nodules there was no evidence of any differences in cell morphology between cells occupied by the Fix^+ or Fix^- strain. If the nodule was unsanctioned (Figure 5A) then all of the cells within the nodule were healthy, regardless of the strain occupying the cell. In contrast, if a nodule was sanctioned (Figure 5B) then – as for a single occupant nodule (Figure 2D) – infected cells within the nodule lost their typical spherical morphology and some burst open. However, this was equally likely to affect cells containing the more or the less effective strain. Therefore, we have seen no evidence to support the cell-autonomous sanctioning.

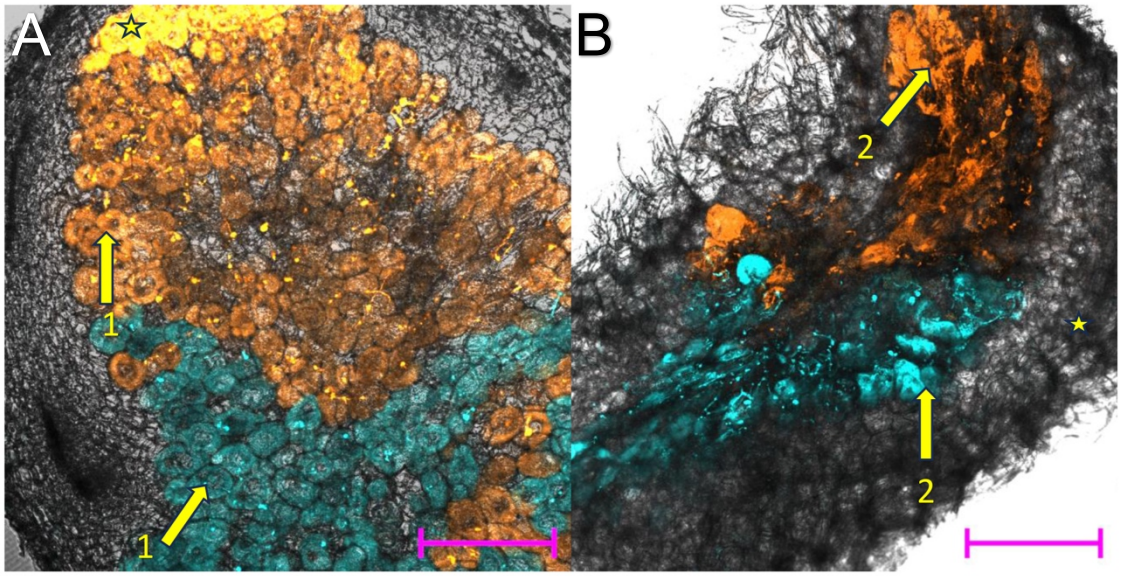

Fig. 5. The two strains within a mixed nodule do not senesce at different times despite varying in relative effectiveness.

Thirty-five days post-inoculation Fix^+ (orange: mCherry tagged) & Fix^- (blue: GFP tagged) mixed nodules (A & B) containing two strains of differing fixation effectiveness. When unsanctioned (A), all cells of both strains remain spherical and intact (1). When sanctioned (B), cells of both strains burst open (2).

Longitudinal nodule slices (100µm) were imaged by confocal microscopy. Scale bar = 200µm. Nodule tip is denoted by star.

3.2.vii Discussion

We show that after twenty-eight days the size of undifferentiated bacteria (Figure 1B) and the number of bacteroids (Figure 1A) but not the number of undifferentiated bacteria (Figure 1C) significantly decreased within a sanctioned nodules. Peas sanctioning nodules containing less effective strains after twenty-eight days agrees with previous studies (Kiers et al., 2003; Oono et al., 2011; West et al., 2002; Westhoek et al., 2017). Consistent with the findings of Westhoek et al., 2021, the number of undifferentiated bacteria did not drop significantly in sanctioned nodules after 28 dpi. Our data is also consistent with conditional sanctioning, as the fate of nodules infected by a Fix^{int} strain depends on whether the co-inoculated strain is Fix^{+} or Fix^{-} (Westhoek et al., 2021). In this study we also used flow cytometry to quantify and analyse the bacteroid and bacterial populations. This revealed that the decrease in size of sanctioned nodules at 28 dpi is linked to a significant drop in the bacteroid population.

Within sanctioned nodules there was also a reduction in the size of undifferentiated bacteria. This suggests that sanctioning causes a reduction of the nutrient supply to the nodule, resulting in bacterial starvation. While bacterial numbers remain high (Fig. 1C), they are much smaller (Fig. 1B), pre-dating the collapse in their population at 56 dpi (Westhoek et al., 2021). Undifferentiated intermediate-fixing bacteria were also significantly smaller when co-inoculated strain with a Fix^{+} strain. While the size of undifferentiated Fix^{-} bacteria changed with the identity of the co-inoculant, the estimated size difference was smaller, and close to the significance threshold.

Compared to unsanctioned nodules, sanctioned nodules have an altered internal structure (Fig. 2). Previous studies have linked these morphological changes to nodule senescence (Regus et al., 2017). By tracking changes through nodule development, we measured the sequence of events that take place throughout sanctioning. Sanctioned nodules are smaller and more spherical by 16 dpi (Westhoek et al., 2017). By 28 dpi there was also a drop in the number of bacteroids and the size of undifferentiated bacteria (Figure 1); however, there was no visible change in the internal structure of sanctioned nodules before 35 dpi (Figure 2). All of these changes appear to precipitate the collapse in undifferentiated bacteria population which is observed by 56 dpi (Westhoek et al., 2021).

It has been proposed that cell-autonomous sanctions occur within mixed nodules (Daubech et al., 2017; Regus et al., 2017). Cell autonomous sanctioning predicts that plant cells containing a less effective bacterial strain will be sanctioned, while plant cells within the same nodule containing a more effective strain will not. If true, then the less effective strain within a mixed nodule should have significantly fewer bacteroids and the size of bacteria should be significantly smaller. Furthermore, we would expect to see premature senescence of plant cells containing the less effective bacterial strain compared to the more effective strain.

Our results did not show any of the above predicted effects within mixed nodules. First, the number of bacteroids was not significantly different (Fig. 3 A). Second, the size of bacteria within mixed nodules was not significantly different (Fig. 3 B). Finally, plant cells within mixed nodules underwent senescence simultaneously, regardless of

effectiveness (Figure 5). Therefore, in peas there is no evidence for cell-autonomous sanctions on the less-effective strain within a mixed nodule.

Whole mixed nodules are sanctioned as an intermediate fixing nodule as we would predict according to conditional sanctioning (Figure 4). This intermediate like sanctioning is to be expected as while the less effective strain will always be sanctioned in a similar manner to Fix^- as seen in Westhoek et al., (2021). There will be a period of time during nodule development where an intermediately fixing nodule will began to fix nitrogen and so will be supported by the plant slightly longer than a Fix^- strain. This additional time allows for the formation of more bacteroids than in a Fix^- nodule, as seen when comparing Fix^- nodules with sanctioned Fix^{int} nodules in Figure 1. Mixed nodules contained fewer bacteroids and smaller bacteria than Fix^+ nodules and significantly more bacteroids than Fix^- nodules. They did not however contain significantly larger bacteria than the Fix^- nodules (Fig. 4). The intermediate level sanctioning of mixed nodules demonstrates that plants can distinguish between nodules of subtly different fixation effectiveness. This supports our conclusion that sanctioning must be controlled through an extraordinarily sensitive response to a plant's overall nitrogen status.

The lack of evidence for cell autonomous sanctions aligns with Agtuca et al., (2020), who demonstrated that the metabolic profile of sectors of mixed nodules containing different strains did not show significant differences. However, our results contrast with studies which have found evidence for cell autonomous sanctions based on variation in the timing of senescence, such as Regus et al., (2017) and Daubech et al., (2017).

The differences may be explained by variation in the legume-rhizobium system used. For example, Daubech et al. (2017) used the *Mimosa pudica* – *Cupriavidus* taiwanensis symbiosis, while Regus et al., (2017) used both the *Acmispon strigosus* – *Bradyrhizobium* and the *Lotus japonicus* – *Mesorhizobium* symbioses. Finally, Agtuca et al. (2020) used the *Glycine max* – *Bradyrhizobium japonicum* symbiosis. A comprehensive understanding of sanctions will therefore require the continued use of multiple experimental systems.

We have shown that the sanctioning of less effective nodules within the pea-rhizobia symbiosis is conditional in nature and occurs at the nodule level. In Fix^{+}/Fix^{int} coinfected plants, nodules containing Fix^{int} are sanctioned while in Fix^{int}/Fix^{-} coinfected plants Fix^{int} nodules are not sanctioned. This suggests that conditional sanctioning requires comparison between nodule-specific and global nitrogen signals. This might be achieved through the interaction between a nitrate receptor such as the Nitrate transreceptor NRT1.1, which both transports nitrate as well as detecting nitrate levels (Zhang et al., 2019) and the NIN-like Proteins (NLPs) 1 and 4, which are essential for the nitrate-based regulation of nodule maturation (Lin et al., 2021). In high nitrate conditions NLP1/4 inhibit cytokinin biosynthesis. Cytokinin biosynthesis drives nodule maturation through the activation of a signal cascade through the Cytokinin responsive element CRE1 (Lin et al., 2021), which acts to promote expression of the *cep* and *cle* genes (Laffont et al., 2020). However, how the nitrogen output of individual nodules is detected by and compared to the global nitrogen status remains unclear. The mechanism of sanctions on individual nodules may be achieved through

nodule-specific proteins responding to nitrogen levels. One possibility is SnRK1 which, when phosphorylated by the DMI2 kinase in response to Nod factor, phosphorylates malate dehydrogenase 1 and 2, leading to increased malate production and supply to bacteroids (Guo et al., 2023). It is therefore a prime candidate for how legumes would reward nodules containing effective strains, as well as to punish nodules containing less effective strains. However, these remain speculations about signalling pathways whose elucidation may aid future efforts to engineer symbioses and in the selection of more effective nitrogen-fixing bacteria.

We now have clear evidence that the change in nodule morphology shown by Westheok et al., (2017) is driven by a reduction in the number of bacteroids and the size of the undifferentiated bacteria. This precedes the changes to the internal structure of sanctioned nodules. The number of undifferentiated bacteria drops at a later timepoint. We have also shown that, while the plant is unable to discriminate between effective and less effective strains within a mixed nodule, ‘piggybacking’ on an effective strain does not provide a viable route by which to evade sanctions. Any potential benefit is limited, because the plant views mixed nodules as less effective, and sanctions them accordingly.

3.3 SUPPLEMENT TO THE PAPER

Carbon transport reporter plasmids

Westhoek et al. (2021) demonstrated that plant sanctions against less effective rhizobial strains are associated with a reduction in the carbon supply. Using biosensors responsive to sucrose and C4-dicarboxylates, the authors showed a marked decrease in luminescence within nodules infected by a Fix^{int} strain at 20 days post-inoculation (dpi). These biosensors, which rely on luminescent reporter systems to indicate the availability of specific carbon substrate, provided clear evidence that sanctioning mechanisms involve, at least in part, a restriction of carbon flow to nodules hosting ineffective symbionts. This carbon deprivation likely contributes to premature senescence and reduced growth of sanctioned nodules, thereby limiting the fitness of less cooperative bacterial strains.

Based on these findings it was hypothesised that if peas were capable of cell sanctioning it would be expected that the cells within a mixed nodule containing a less effective fixer would receive a reduced supply of carbon from the plant. To this end, the luminescence-based carbon biosensor system used by Westhoek et al. was adapted to a fluorescence-based system that would be compatible with spatially resolved imaging techniques such as confocal laser scanning microscopy. This adaptation was necessary because luminescent signals are difficult to spatially localise within tissue, whereas fluorescent proteins allow for high-resolution imaging of cellular processes within tissue sections. Specifically, the luminescence reporter

was replaced with a fluorescent protein, generating a reporter plasmid in which the *pdctA* promoter drives expression of mTagBFP (Figure 6).

This plasmid was introduced into the pre-existing rhizobial strains: a Fix^{+} strain carrying a constitutive mCherry marker, and a Fix^{int} strain carrying constitutive GFP. Nodules were harvested at 28 dpi and sectioned imaging via confocal microscopy, allowing for visualisation of the spatial distribution of the *pdctA*-driven mTagBFP reporter within sectioned nodules. The goal was to assess whether differential expression of the carbon biosensor could be detected in cells infected by the more effective versus the less effective strain, particularly within the same nodule.

However, this approach was not effective for resolving differences in carbon allocation at the level of individual infected plant cells. Across all nodule types examined (fully effective (Figure 6A), fully ineffective (Figure 6B), and mixed (Figure 6C)) the expression of the *pdctA*-driven reporter appeared broadly consistent across the different infection types, with no obvious spatial patterns or differences in signal intensity that could be correlated with the effectiveness of the infecting strain.

The failure of the mTagBFP reporter to replicate the results of Westhoek et al., (2021) may be attributed to a number of potential issues. The first is that the stability of the mTag fluorescent protein may lead to an accumulation of the protein prior to 20 dpi, when the significant change in luminescence was seen in Westhoek et al., (2021). While there is then a significant drop in the rate of carbon transport into the nodule at this point the stability of the existing fluorescent protein results in no differentiation between a sanctioned or unsanctioned nodule. The second possibility is that the

plasmid reporter is leaky leading to effectively constitutive expression. This is potentially the more likely of the two possibilities as pOPS1956 contains a particularly strong Ribosome binding site RBSstd.

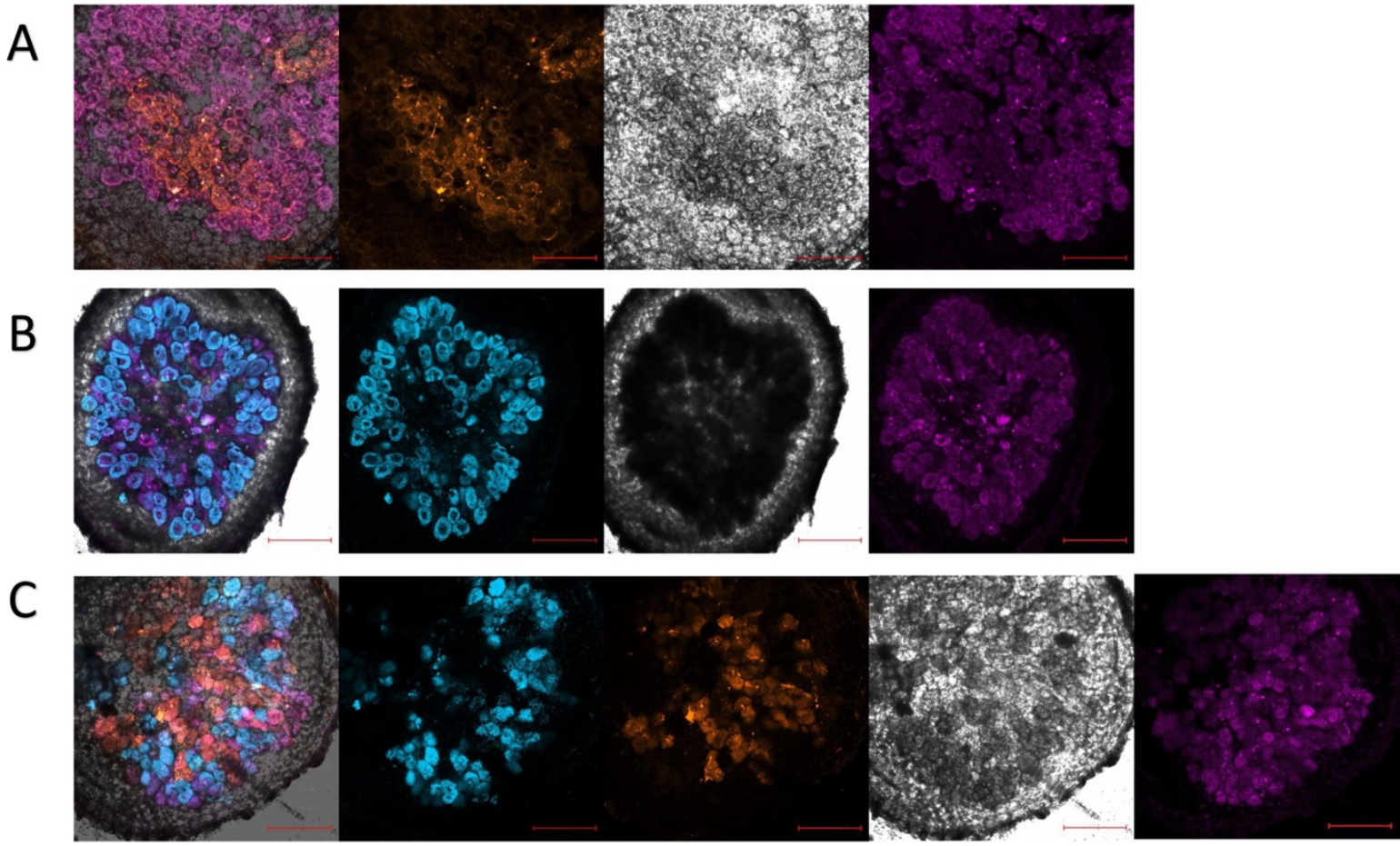

Fig 6. A p*dcta* fluorescent biosensor is ineffective at visualising changes in Carbon transport

Peas were inoculated with fluorescently tagged Fix^+ (orange: mCherry) & a Fix^- (blue: GFP) strain. These strains also carried a p*dcta* mTag BFP reporter (violet). Nodules containing the Fix^+ strain (A), Fix^- strain (B) or both (C) were sectioned and visualised using confocal microscopy.

Longitudinal nodule slices (100µm) were imaged by confocal microscopy. Scale bar = 200µm.

3.4 CONCLUSION

This study provides clear evidence that *Pisum sativum* applies sanctions against less effective strains at the level of the whole nodule but does not apply them to individual infected plant cells. Through an integrated approach combining flow cytometry and confocal microscopy, it has been shown that sanctioning in pea plants manifests as a significant reduction in the number of bacteroids and the size of undifferentiated bacteria within nodules. This reduction in size may explain why there is no drop in the number of bacteria by 28 dpi, it may be theorised that the carbon starvation present from 20 dpi has led to this change in bacteria size but has not as yet reduced the population size. These phenotypes are accompanied by early-onset morphological changes in infected cells which likely prelude early senescence of the nodule, reinforcing the idea that host plants impose resource limitation as a mechanism to reduce the fitness of ineffective symbionts.

Crucially, analysis of mixed nodules, those simultaneously infected with both effective and ineffective strains, revealed that sanctioning is not cell-autonomous. Within these nodules, there were no significant differences in bacterial size, number of bacteroids, or cell morphology between plant cells hosting more or less effective strains. Instead, the plant appears to sanction the nodule as a whole, based on overall nitrogen output. This results in mixed nodules receiving an intermediate level of sanctioning compared to single-strain nodules. This finding is consistent with a model where the host assesses and responds to nitrogen fixation at the nodule level. The intermediate level

sanctioning of mixed nodules does however make piggy-backing a potentially unrealistic route for less effective strains to prosper.

Attempts to spatially resolve differences in carbon allocation using a fluorescent reporter under control of the carbon-responsive *pdctA* promoter were inconclusive, likely due to reporter stability and leaky expression. This prevented a visual demonstration of the restriction in resource allocation shown in Westhoek et al (2021). However, this work was ultimately not necessary in efforts to test the theory of cell-sanctioning as other schemes of work have provided strong evidence against this theory.

In summary, this work robustly demonstrates that sanctioning in pea-rhizobium symbiosis is conditional and nodule-level rather than cell-specific. These findings have important implications for our understanding of mutualism stability and the evolutionary dynamics of symbiotic nitrogen fixation.

Following on from this work a key question remaining was how less-effective strains have remained prevalent in ‘wild’ rhizospheres. It was therefore decided that the next section of this project would be focussed on understanding the short falls of sanctioning and what, if any, avenues exist that may allow a less effective strain to evade the associated fitness costs.

4: Strategies for the evasion of host sanctions

4.1: Introduction

In Chapter 3 it was shown that piggy-backing, entering a nodule simultaneously with a more effective fixer, is not a realistic route by which to evade sanctioning as mixed nodules are overall sanctioned. It is well documented however that wild rhizobia do show a significant degree of variation in fixation effectiveness between strains (Berrada et al., 2019; Woliy et al., 2019). As such there must be some mechanism by which a less effective fixer can at least substantially mitigate the fitness costs of sanctioning. The reasoning being that if this was not the case the expectation would be for less effective fixers to be consistently and heavily punished leading to the dominance of effective fixers and relative uniformity in fixation effectiveness in wild strains.

Therefore, the next goal of this project was to explore potential avenues by which a less effective strain may evade sanctions and remain prevalent in wild populations. To begin this scheme of work the first step was to use the existing understanding of the symbiosis in order to hypothesise scenarios in which either the host-plant would be unable to differentiate between strains or where it would be deleterious to the host-plant to enforce sanctions normally. Having shown, in chapter 3, the effects of sanctions on the inhabitants of a sanctioned nodule the following work utilised the simpler approach taken in other papers and measured the size of nodules as a proxy for sanctioning (Westhoek et al., 2017).

Westhoek et al., (2017) demonstrated that peas do not exhibit partner choice in their infection by symbionts varying in fixation effectiveness. Therefore, the host plant cannot influence the proportion of nodules occupied by a more or less effective strain. This means that if the population of rhizobia in the rhizosphere is predominantly made up of a less effective fixer, or if a less effective fixer develops the ability to nodulate the host-plant more effectively, then the proportion of nodules occupied by a less effective fixer will exceed fifty percent. In fact, there appears to be no mechanism in place to prevent this strain occupying a large proportion or even all of the nodules on a plant.

If a less effective fixer was to occupy 80-90% of nodules would it still be profitable for the host-plant to apply sanctions normally? At some point the number of nodules occupied by the more effective fixer is so low that just rewarding these nodules will not provide sufficient nitrogen to meet the plant's needs. Two outcomes may then occur i) the plant continues to apply sanctions uniformly resulting in nitrogen starvation and reduced fitness of the host plant or ii) the host-plant attenuates the severity of sanctions leading to a less effective fixer being rewarded. In the second scenario it is clear that this less effective fixer has evaded the consequences of sanctioning. This first avenue for cheats was tested by observing the treatment of nodules of a less effective fixer as the proportion of nodules occupied by the strain increases.

Westhoek et al., (2021) and the work in chapter 3 of this thesis used the same Fix^{int} strain. This strain fixes at approximately 50% the rate of the wild-type Fix^{+}. The variation in fixation therefore is 50%. The strains examined in the wild rhizobia studies did not vary by quite as extreme a degree (Berrada et al., 2019; Woliy et al., 2019). It is possible that as the difference in fixation effectiveness decreases the ability of the host plant to differentiate between them is compromised.

To test this theory a strain who's effectiveness was between the Fix^{+} and Fix^{int} strains was needed. While searching through the literature a candidate strain was found in Rutten et al., (2021) in which a strain mutated in its oxygen sensing pathway had been characterised and was found to have significantly reduced fixation relative to wild-type 3841 (Fix^{+}). This strain was a double knock-out mutant of the two copies of *fixL* (C & 9) which are required for oxygen sensing within the symbiosome. Under hypoxic conditions FixL via intermediaries' drives expression of the *fix*NOQP operon which is required for respiration and ATP production under low oxygen conditions. Therefore, the *fix*L mutant is no longer able to respire effectively and properly fuel nitrogen fixation. This strain was shown in Rutten et al., (2021) to fix nitrogen at approximately 70% of wild-type.

Conditional sanctioning has demonstrated that the host plant is capable of comparing the nitrogen output of its nodules. It is conceivable that this comparison occurs on a local level in which nodules that are proximal to each other are compared. If this was the case, then a less effective nodule that is spatially separated from a more effective nodule would not be sanctioned or would be sanctioned less heavily than a nodule

that is proximal to a more effective nodule. Alternatively, these comparisons may occur globally i.e. the outputs of all nodules are compared with the outputs of all others across the root system.

It is also possible that a combination of these two takes place. If there is any element of the comparison that occurs locally then a less effective strain may evade sanctions by dominating nodulation within a sub section of a root system. To test for global and/or local elements of a sanctioning a split root system was established. By bifurcating the root system it was possible to expose spatially separated sections of root to different inocula. Using the Fix^{+} and Fix^{int} strains added separately or in combination to split roots the effect of proximity or separation on sanctioning could be tested.

In this chapter I aimed to find which factors influenced the application or severity of sanctions as well as what comparisons the host plant was capable of making both in terms of distance between nodules as well as fixation effectiveness. The ultimate goal being to use this greater understanding to guide the work carried out for chapter 5 by narrowing down the list of potential regulators to those that were consistent with the findings of chapter 3 and 4.

4.2: RESOURCE ALLOCATION IN THE NODULES OF THE *PISUM SATIVUM* – *RHIZOBIUM* SYMBIOSIS

4.2.i Forward

The work in this chapter is presented in the form of a manuscript submitted for publication, is currently undergoing peer-review, and is available as a preprint (Underwood et al., 2025). Minor formatting changes have been made to fit the format of this thesis. The supporting information for this paper has been provided in Appenix B. The reference list has been moved and combined with the thesis bibliography.

4.2.ii List of supporting information

Fig. S1: R markdown file for output of statistical anlaysis

Doc. S2: Spreadheet of data collectd and analaysed for this manuscript

4.2.iii Abstract

Legumes host nitrogen-fixing bacteria, called rhizobia, within specialised root structures called nodules, where carbon from the plant is exchanged for ammonia fixed from N_2 by the bacteria. Legumes can host multiple bacterial strains at the same time that vary in their fixation effectiveness but legumes sanction nodules containing less effectively fixing strains by reducing the provision of nutrients. Understanding how sanctions are applied by plants and how bacteria may try to avoid them is important to understand the stability of legume-rhizobial symbioses. Using near isogenic *Rhizobium leguminosarum* strains on pea we demonstrate that sanctions are sensitive

to the proportion of nodules occupied by a less effective strain and by using split roots show that sanctions are applied based on global comparisons of nodules across the plants root system. By using several rhizobia with different levels of fixation, but all derived from the same parent, we show that pea plants can differentiate between bacteria with relatively small variations in fixation effectiveness. We demonstrate that peas integrate global and local signals in order to determine whether individual nodules are sanctioned. At the same time these results show that poorly fixing strains can avoid sanctions if they dominate nodulation.

4.2.iv Introduction

Legumes, such as *Pisum sativum* (pea), can establish a mutualistic symbiosis with nitrogen-fixing bacteria known as rhizobia (Poole et al., 2018). In nitrogen-limited environments, this association allows the host plant to access a biologically usable form of nitrogen. Central to this symbiosis is the formation of root nodules, specialised organs within which rhizobia differentiate into Y-shaped bacteroids capable of fixing atmospheric nitrogen into ammonia (Mergaert et al., 2006). In exchange for fixed nitrogen, the host supplies carbon, primarily in the form of C4-dicarboxylates. This reciprocal exchange of nutrients underpins the mutualistic nature of the interaction (Udvardi & Poole, 2013).

These nodules provide a highly advantageous niche for rhizobia, enabling them to multiply to high densities before being released back into the soil upon nodule senescence (Timmers et al., 2000). However, this ecological opportunity opens the

door for ‘cheater’ strains: rhizobia that colonise nodules and benefit from carbon and amplification without investing in nitrogen fixation (R. F. Denison, 2000).

Previous studies (Underwood et al., 2024; Westhoek et al., 2017, 2021) have demonstrated that built into this symbiosis on the part of the host plant is a sanctioning mechanism capable of punishing any ‘cheating’ bacteria that occupy a nodule. These ‘cheaters’ could potentially gain a fitness advantage by occupying a nodule, benefitting from the supply of carbon and amplification of numbers while expending less energy on nitrogen-fixation, thereby gaining a fitness advantage over a non-cheating strain.

Westhoek et al. (2021) showed that peas apply sanctions to nodules containing a strain that is less effective at fixing N_2 by reducing their carbon supply. Remarkably, these sanctions are not applied to nodules based on a set threshold value of fixation. Instead, peas conditionally sanction a strain based on the other strains present. In this way a mediocre fixing strain may be sanctioned when a highly effective fixing strain is present but remains unsanctioned when a less effective strain is present.

This dynamic form of sanctioning raises important questions about the evolutionary stability of the symbiosis and the potential for exploitation by less cooperative strains. It has been shown that rhizobia of varying fixation effectiveness are present naturally (Berrada et al., 2019; Woliy et al., 2019), therefore, a strategy must exist through which less effective strains prosper. In this study, we investigated three possible avenues through which strains with reduced nitrogen fixation effectiveness might evade host sanctions and gain a competitive advantage:

1. **High Proportion of Cheaters:**

   In natural settings, rhizobial populations are unlikely to infect hosts in a 1:1 ratio. Since peas lack a strong 'partner choice' mechanism (Westhoek et al., 2017), they may be colonised by highly uneven ratios of effective and ineffectively fixing strains. If most nodules are occupied by low-fixing strains, stringent sanctioning may threaten the plant's nitrogen supply. If, instead, the plant relaxes sanctions this may result in the increased abundance of the cheating strain. We tested this by inoculating peas with different ratios of a highly effective and a less effective strains and measuring sanction responses.

2. **Local Clusters of Cheaters:**

   Conditional sanctioning implies some form of comparison between nodules. Whether this occurs locally (between spatially proximate nodules) or globally (across the entire root system) remains unknown. If sanctions are applied based on local comparisons, a less effective strain might avoid punishment by clustering with similar nodules. To assess this, we employed a split-root system to spatially separate strains and evaluate nodule-specific responses.

3. **Smaller Differences in Fixation Effectiveness:**

   To date, studies have compared strains with relatively large differences in fixation capacity (Westhoek et al., 2021). However, it remains unclear how finely tuned is the ability of plants to discriminate between strains. If the resolution is coarse, a strain with only slightly reduced fixation could avoid sanctions. To test this, we introduced a third strain, a *fixL* double mutant

(Rutten et al., 2021) which fixes nitrogen at a level intermediate to the previously tested Fix^{+} and Fix^{int} strains from Westhoek et al., (2021). This allowed us to assess the sensitivity of the sanctioning system of plants to subtle differences in symbiont performance.

Understanding how less effective or 'cheating' strains might evade host control is crucial for explaining the evolutionary dynamics of the legume rhizobia symbiosis and for maximising the continued benefit of the symbiosis to modern agriculture. The scattered, paraphyletic distribution of nodulation within the nitrogen-fixing clade of Rosids (Doyle, 2016), including partial losses within the Fabaceae, raises the possibility that uncontrolled symbiont exploitation may contribute to the loss of nodulation in some lineages.

Our findings demonstrate that peas modulate the severity of sanctions based on the relative abundance of strains, employing a global rather than a local mechanism of comparison. Additionally, peas can detect finer gradations in fixation effectiveness than previously shown, although an even smaller difference may still provide a loophole for cheating strains. These results refine our understanding of host control mechanisms and their limitations, with implications for the stability and breakdown of mutualism in this important symbiosis.

4.2.v Methods

Rhizobial Strains and Culture conditions

The strains used in this work are all derived from an effective nitrogen-fixing bacteria Rhizobium leguminosarum bv. (Rlv) 3841 that is a root symbiont of *Pisum sativum* L. cv. Avola (pea) (Johnston & Beringer, 1975). The strains used varied in their fixation ability or fluorescent tag but are otherwise isogenic (Table 1). Some of the strains were tagged with a fluorescent marker (mCherry, GFP) to distinguish between the nodules formed by each strain (Table 1). Strains were maintained on tryptone-yeast (TY) agar (Beringer, 1974) with the required concentrations of antibiotics (Table 1). For longer-term storage a solution of TY with 15-20% glycerol was inoculated and then stored at -80°C. For inoculation of peas rhizobia were grown on a TY agar slope. The slopes were then washed using UMS and the number of bacteria present in the medium was determined by measuring OD600 using a Genesys 250 UV-Visible spectrophotometer. This solution was then diluted into a solution of approximately $1x10^{7}$ ml^{-1}.

Plant Growth

Peas were surface sterilised (1 minute in 95% ethanol followed by 5 minutes in 20% NaClO), rinsed with sterile water and left to germinate for five days on 1% w/v agar plates at room temperature in the dark.

Single pot experiments

After the five days of germination seedlings were transplanted to a sterilized 500 ml Azlon beaker for competition assays and 1 L Azlon beaker for Acetylene Reduction Assays. Beakers contained a 1:1 Mixture of silver sand and fine vermiculite and sterilized nitrogen-free nutrient solution (75 ml for 500 ml beakers and 150 ml for 1 L beakers). For single strain experiments $1x10^7$ cells of the desired rhizobial strain were added. For co-inoculated experiments the desired ratio (1:1 – 9:1) of the two rhizobial strains was added ($0.5x10^7$ cells of each) (See Westhoek et al., (2017)). Beakers were covered with clingfilm to prevent aerial contamination. This was slit after a few days to allow seedlings to grow through. Plants were grown in a growth chamber (21 °C, 16 h photoperiod) for 28 days and watered as necessary from 7 days onwards.

Split root experiments

After five days of germination the primary root of the seedling was cut laterally using a sterile scalpel, removing the tip of the root below where lateral root hairs had begun to form. These cut seedlings were then moved onto fresh 1% w/v agar plates, the edges of the plate were sealed with micropore tape and then placed in a growth chamber (21 °C, 16 h photoperiod) for a further five days. Seedlings were then placed across two 500 ml Azlon beakers with approximately half of the newly formed lateral roots placed into each beaker. The beakers contained a 1:1 Mixture of silver sand and fine vermiculite and 75ml of sterilized nitrogen-free nutrient solution. To each pot a total of $0.5x10^7$ rhizobial cells was added of the desired strain/strains. Plants were grown in a growth chamber (21 °C, 16 h photoperiod) for 28 days. The exposed root

split across the pots was lightly watered every day for the first 7 days and then watered as necessary. In split root experiments a 1:1 ratio of each strain was always applied to the plant as whole.

Harvesting

Plants were harvested 28 days post inoculation (dpi). Plants were removed from the sand vermiculite mix and washed carefully. Nodules were then imaged using a LEICA M165 FC fluorescent stereo microscope and an iBright FL1500 imaging system. Nodule occupants were identified based on their fluorescence (Fix^{+} tagged with GFP & Fix^{int} tagged with mCherry) or the lack of fluorescence (Fix^{L} untagged). The nodules of each strain were counted either per plant for single beaker experiments or per beaker for split root experiments.

Nodule measurement

The five largest nodules of each strain on each plant or side of split root were selected by eye and then picked and photographed. These nodules were then measured using the Fiji analysis software (v2.14.0/1.54f). A ruler was included in each photograph of the nodules so that the scale for each image could be set by drawing a line of 10 mm over the markings of the ruler using a straight-line selection. Each nodule was then drawn around using the polygon selection tool and the area of the region of interest was measured. The mean of the five nodule sizes was calculated and taken as the average nodule size.

Acetylene Reduction Assays

For acetylene reduction of experiments the whole plant was placed directly into a Schott bottle. The acetylene reduction assay was then carried out using the method described in Westhoek et al. (2021). Fixation effectiveness was then calculated as a percentage of the average fixation rate of Fix^+. Fix^+ therefore fixed at 100%, Fix^L fixed nitrogen at approximately 70% the rate of Fix^+ and Fix^{int} fixed nitrogen at approximately 40% the rate of Fix^+ (Fig. 1).

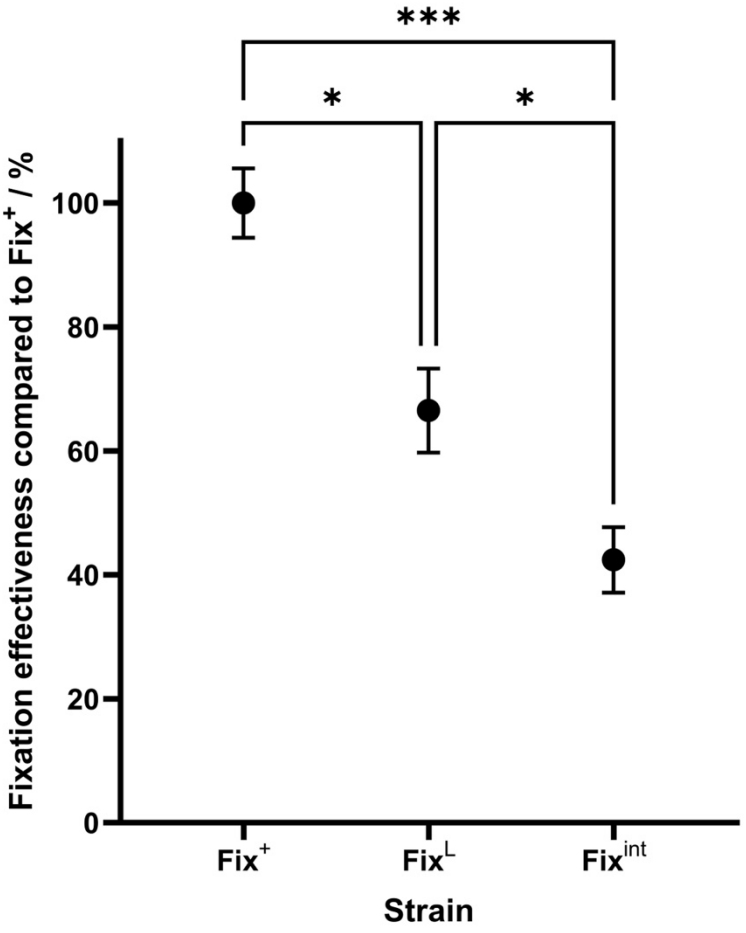

Fig. 1: Fixation effectiveness of three near isogenic strains

The rate of fixation was measured for three strains. The values were converted to a percentage of the average fixation rate of Fix^+ (wild-type Rlv 3841). Black dots denote average fixation effectiveness. Error bars are one standard error of the mean. Statistical comparison carried out though one-way ANOVA and Tukey's post-hoc test. * = $p < 0.05$, *** = $p < 0.001$.

Statistical analysis

Fix^{+} vs Fix^{int} single pot co-inoculation:

To test the correlation between the proportion of a strain within the inoculum and the proportion of nodules containing a strain a linear regression analysis was employed. A linear regression was also used to assess the correlation between the number of nodules a strain occupied and the average size of the nodules containing that strain.

Fix^{+} vs Fix^{int} split root co-inoculation:

When testing for a difference in average size between Fix^{+} & Fix^{int} nodules in a split root system a nested linear mixed effect model was used in which the experiment number was a random factor and the plant number was used as a grouping factor allowing a pairwise comparison of nodules on the same plant across our biological replicates. As in the single pot experiments, when assessing the correlation between the number of nodules a strain occupied and the average size of those nodules, a linear regression was used

To test for a significant difference in the size of nodules on a split root plant when inoculated into separate pots or mixed into both pots a nested linear mixed effects model was used with experiment number as a random factor and plant as a grouping factor for pairwise comparison. This model was then tested using a one-way ANOVA, if the ANOVA showed a significant difference between groups this was then followed up with a Tukey's post-hoc test.

Fix^{+}, Fix^{int} & Fix^{L}

To test the effect of co-inoculum on three strains of varying fixation effectiveness a linear model was produced for each strain comprised of the size of the nodules containing the strain and the co-inoculum (either one of the other two strains or the same strain). A one-way ANOVA was then used to test whether co-inoculum led to a significant variation in nodule size. If this test returned a significant difference, then a Tukey's post-hoc test was used to compare each of the co-inoculum to the others.

All statistical analysis was carried out using R version 4.4.1 (2024-06-14)) and RStudio (2022.02.3+492 "Prairie Trillium"). Graphs were produced using Graph Pad version 9. An R markdown document is provided (Fig. S1). The model assumptions of equal variance and normality were assessed by visual inspection of residual plots (see R Markdown). Values from statistical tests are given where a significant trend or difference was found ($p < 0.05$). Where no significant trend or difference was found ($p > 0.05$) the values are not given. All statistical test outputs are provided in the R Markdown (Fig. S1). Data for each experiment is provided in Doc. S2.

Table 1: Rhizobial strains

All strains derived from Rlv3841 and provided with a strain code, resistance markers, short description and reference.

Name	Strain	Antibiotic resistance	Description	Reference
Fix^{+}	Rlv3841	strep	Rlv3841	(Johnston & Beringer, 1975)
Fix^{+} GFP	OPS1339	Strep, GM	Rlv3841 Tn7-Gm- GFP	(Westhoek et al., 2021)
Fix^{int} mCherry	OPS2269	Step, Spec, GM,	Fixint Tn*7*-Gm-mCherry (Rlv3841 mutant, ΩSpc cassette in promoter region of *nifA*)	(Westhoek et al., 2021)
Fix^{L}	LMB496	Strep, spec,	$hfixL_9$ :: omega spec, $hfixL_c$:Pk19	(Rutten et al., 2021)

4.2.vi Results

The severity of sanctioning is dependent on the proportion of cheats

To maximise the acquisition of nitrogen from nodule fixation legumes punish cheating strains. However, when the ratio of cheating to effective strains is varied it may be hypothesised that in order to still meet the plants nitrogen requirements it would be advantageous for the legume to relax sanctions as the proportion of cheaters

increases. By the same logic it would also be expected that sanctions would become more severe as the proportion of cheaters decrease.

To test the effect of varying the frequency of the cheating strain, peas were inoculated with Fix^+ (GFP) and Fix^{int} (mCherry) at varying ratios (1:9, 1:2, 1:1, 2:1, 9:1). As shown in (Westhoek et al., 2017) there was a significant linear correlation between a strains proportion of the inoculum and the proportion of nodules occupied by the strain ($y = 0.99X – 1$, $R^2 = 0.961$, $p < 0.0001$)(Fig. 2 A). The average size of the nodules containing the less effective Fix^{int} was significantly positively correlated with the frequency of nodules containing Fix^{int} ($y = 30X – 16$, $R^2 = 0.731$, $p < 0.0001$)(Fig. 2 B). In contrast, the size of nodules containing the more effective Fix^+ strain did not show a significant correlation to the number of nodules the strain occupied (Fig. 2 C).

From this result it may be concluded that, as hypothesised, the severity of sanctions imposed on a less effective strain varies with the strains relative frequency. At the same time this was not true for the more effective strain. This suggests that the more effective strain is always receiving the maximum benefit from the host plant and the legume will then vary its treatment of the less effective strain to maximise its supply of nitrogen from nodules.

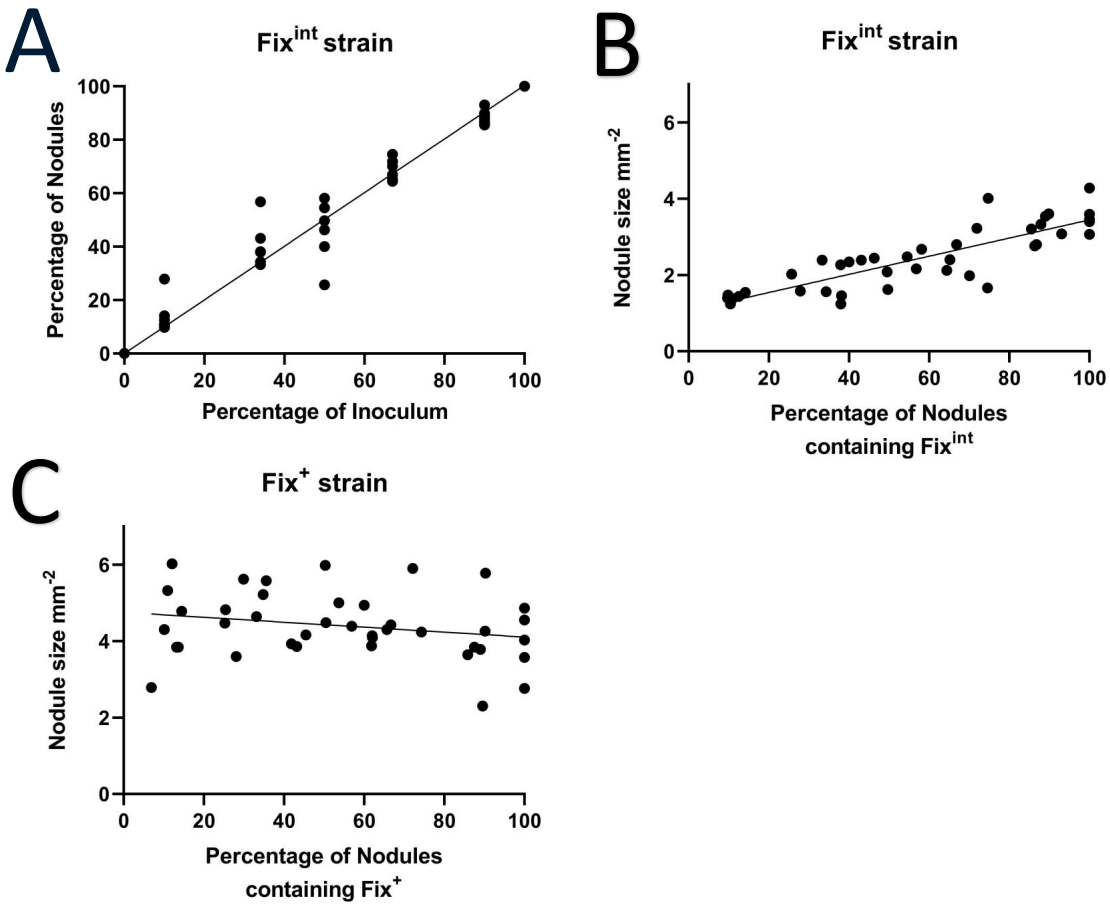

Fig 2. The sanctioning of a less effective nodule is dependent on their proportion of total nodules

For peas inoculated with a Fix^+ and Fix^{int} strain, the percentage of nodules containing a Fix^{int} strain and the percentage of the Fix^{int} strain within the inoculum applied (A). The average size of the Fix^{int} (B) and Fix^+ (C) containing nodules on these plants plotted against the percentage of nodules containing the relevant strain. Each point represents the percentage (A) or the average size (B & C) for one plant. Trend line is a linear regression.

Sanctioning is based on a global comparison between nodules

To test to what extent sanctioning is controlled through a global or local signalling mechanism a split root system for pea was developed, allowing the root system of a single pea plant to be inoculated with two different strains in physically separate pots thereby eliminating any potential effect of a local signalling mechanism between nodules that are proximal to each other.

When spatially separated, Fix^{+} nodules were significantly larger than the Fix^{int} nodules (est. = 3.4191, SE = 0.1717, df = 9, t = 19.909, $p < 0.0001$) (Fig. 3A). When comparing the size of the Fix^{int} nodules with the proportion of nodules occupied by Fix^{int} these plants also showed the same pattern seen in the single pot experiments i.e. as the proportion of the less effective strain increased so did the size of the nodules containing the less effective strain ($y = 13X + 26$, SE = 3.912, $R^2 = 0.531$, $p = 0.0101$) (Figure 3B).

This result demonstrates that the effect of sanctions is not altered by spatial separation of the nodules. Therefore, peas carry out a global comparison between the nodules present and apply sanctions accordingly

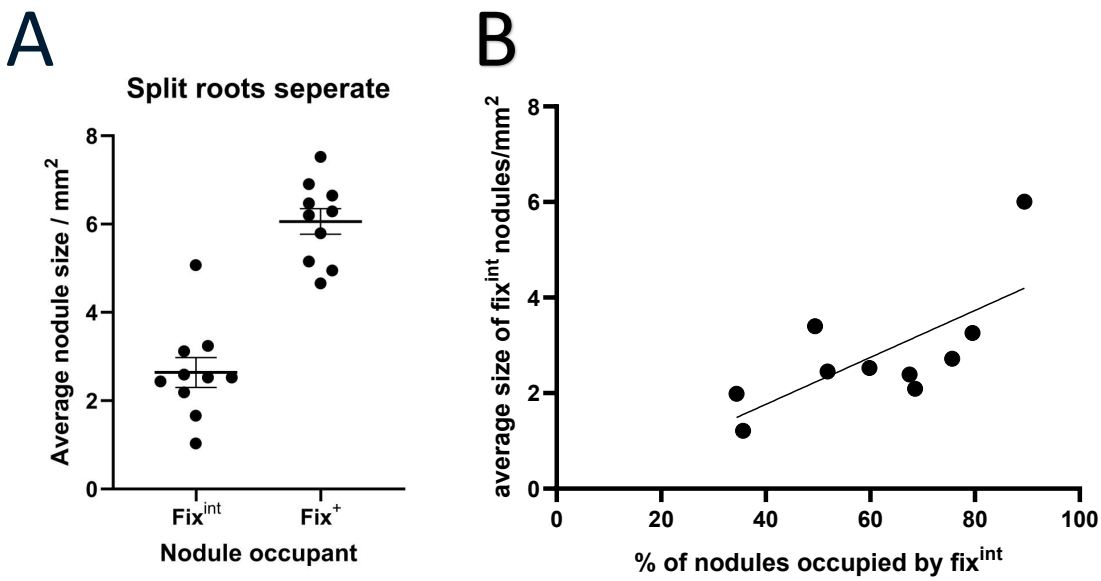

Fig 3. Split root peas still impose sanctions

Peas with split roots placed across two pots were inoculated with a Fix^+ strain and a Fix^{int} strain with each strain added to a separate pot. The average size per plant of the nodules for each strain was measured (A). The relationship between the proportion of nodules occupied by the less effective Fix^{int} and the size of those nodules was also compared (B). Each point indicates the average value for a single plant. For B the trend line is a linear regression.

Proximity does not alter sanctioning

It is conceivable that there is a local as well as a global element to the application of sanctions. To test this a series of split root plants were inoculated with both the Fix^{+} and Fix^{int} strain into both pots. These mixed rather than separately inoculated split root plants could then be compared to the separately inoculated plants. If there was a proximity effect on sanctioning it would be expected that the severity of sanctioning would significantly vary between these two sets of split root plants.

The Fix^{int} nodules on split roots with mixed inoculum were significantly smaller than the Fix^{+} nodules on the same plants (estimate = 3.2811, SE = 0.1642, df = 8.99, t = 19.98, $p < 0.0001$) (Figure 4A). When comparing the nodules on the separately inoculated split roots to those on the mix inoculated plants there was no significant difference between the two sets of Fix^{+} nodules or the two sets of Fix^{int} nodules. As was the case for both the single pot and the separately inoculated split root plants the size of the Fix^{int} nodules was significantly correlated with the proportion of nodules occupied by the Fix^{int} strain ($y = 14.2X + 25$, SE = 4.783, $R^2 = 0.465$, $p = 0.018$) (Figure 4B). In short, the results of the mix inoculated split root plants were identical to those of the separately inoculated split roots.

These results demonstrate that the treatment of neither the more nor the less effective strain on a split root plant varies significantly due to the proximity of nodules containing a different strain. We therefore conclude that under optimal conditions sanctioning is regulated by a global signalling mechanism that is unaffected by local cues. However, in field conditions where biotic or abiotic stresses may be very local it may be required

for the host to make local instead of global decisions e.g. local nitrate levels may shift the equation as to whether a nodule, regardless of occupant is worthy of investment.

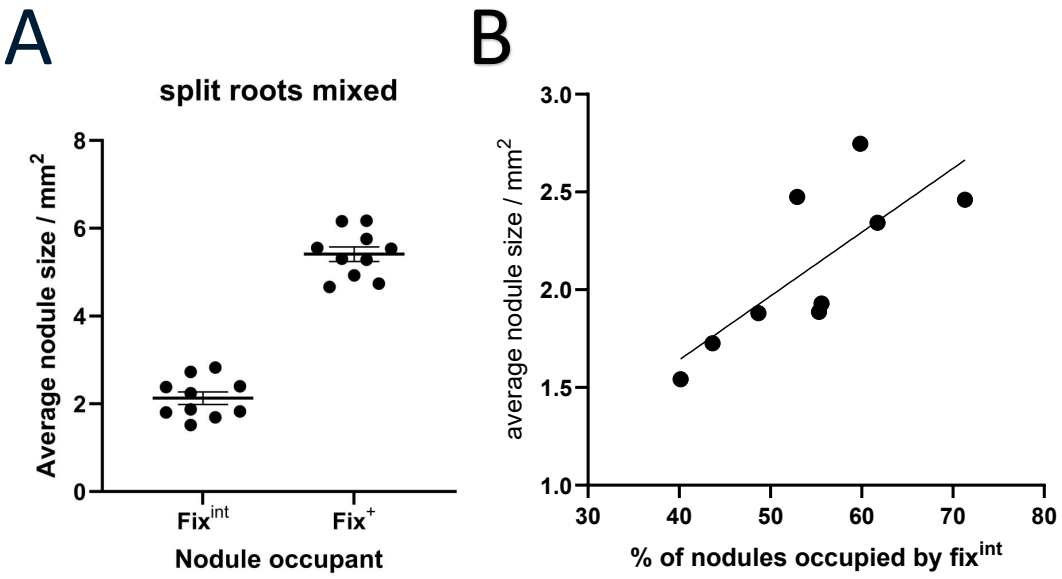

Fig 4. Sanctions are not modified by proximity

Peas with split roots placed across two pots were inoculated with a 1:1 mix of a Fix^+ strain and a Fix^{int} strain added to each pot. The average size per plant of the nodules for each strain was measured (A). The relationship between the proportion of nodules occupied by the less effective Fix^{int} and the size of those nodules was also compared (B). Each point indicates the average value for a single plant. For B the trend line is a linear regression.

Peas can detect smaller changes in fixation effectiveness

To test the ability of peas to detect smaller differences in fixation effectiveness plants were inoculated with one of three combinations of strains. The Fix^{+} & Fix^{int} strains used in Westhoek et al., (2021) and Underwood et al., (2025) as well as each of these strains with the Fix^{L} strain from Rutten et al., (2021). This Fix^{L} strain fixes at a rate in between that of the Fix^{int} and Fix^{+} strain (Fig. 1) and therefore the differences in fixation between the Fix^{L} strain and the others is smaller than between Fix^{+} and Fix^{int}.

When Fix^{L} was co-inoculated with Fix^{+} the nodules containing Fix^{L} were significantly smaller than when Fix^{L} was the sole inoculant (estimate = -1.889, 95% CI = -3.518 : -0.258, p = 0.023) or when Fix^{L} was co-inoculated with Fix^{int} (estimate = -2.450, 95% CI =) (Fig. 5A). Nodules containing the Fix^{int} strain were significantly smaller when co-inoculated with Fix^{+} (estimate= = -2.136, 95% CI = -4.010 : -0.889, p = 0.003) or Fix^{L} (estimate= = -1.516 95% CI = -2.807 : -0.226, p = 0.021) compared to when Fix^{int} was the sole inoculant (Fig. 5B). Fix^{+} nodules did not significantly vary in size regardless of co-inoculant or when solo inoculated (Fig. 5C). The sanctioning of Fix^{int} when co-inoculated with Fix^{L} and the sanctioning of Fix^{L} when co-inoculated with Fix^{+} indicates that the host plant was capable of differentiating between the three strains and successfully punishing the less effective strain.

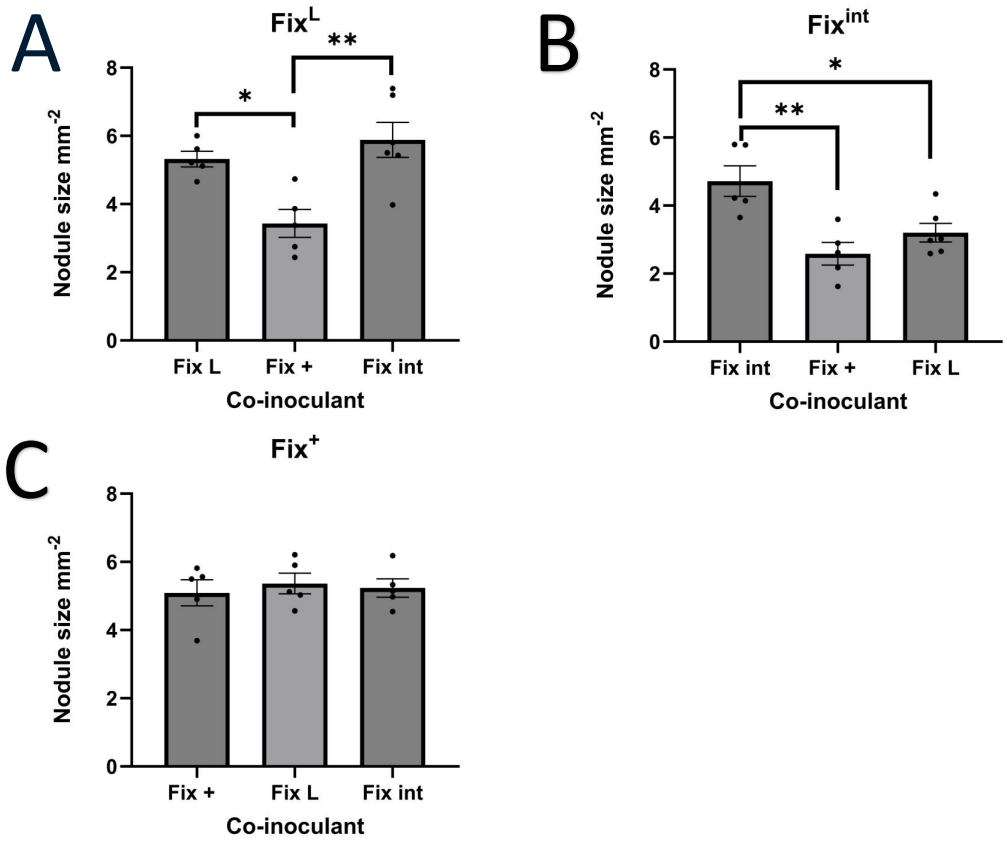

Fig. 5 Peas can detect small variations in fixation and apply sanctions accordingly

Peas were inoculated with a Fix^{L} (A), Fix^{int} (B) or a Fix^{+} (C) strain. These strains were co-inoculated either with themselves or with one of the other two strains. Nodules were harvested after 28 days and average nodule size was measured. Each black point indicates the average nodule size for one plant. Bars indicate the mean of the average nodule sizes. Error bars indicate the standard error of the mean.

4.2.vii Discussion

We have shown that peas moderate the severity of the sanctions imposed on a less effective strain depending on the proportion of nodules occupied (Fig. 2). We also conclude that peas compare nodules across their root system through a global signalling mechanism (Fig. 3) that does not include a local comparison between nodules that are proximal to each other (Fig. 4). We have tested the ability of peas to differentiate between smaller variations in fixation effectiveness than has been previously tested and found that they were still able to sanction the less effective strain (Fig. 5). However, this result does not allow us to make a broader conclusion on the ability of a less effective strain with a smaller variation in fixation effectiveness to evade sanctions.

This study has tested three avenues for cheating strains to prosper in a diverse population of nitrogen-fixing bacteria and has concluded that a viable avenue does exist. If a cheating strain dominates nodulation, then the plant will reward the strain. This is likely because when such a large number of nodules are occupied by a less effective strain the plant is unable to meet its nitrogen requirements by only rewarding the more effective strain. Thereby forcing the plant to reduce or entirely suspend sanctioning of the less effectively fixing strain. This result partially explains the inability of conditional sanctioning to eradicate less effective strains in wild populations. When considering the use of effectively fixing inoculum to boost yields in an agricultural setting it is therefore crucial to consider the relative size of the population and the competitive nodulating ability of the strain applied relative to the pre-existing strains in

the soil. If too low a population of the introduced strain is present or if the strain cannot nodulate competitively then too few nodules will be occupied by the effective fixing strain allowing less effectively fixing strains to evade sanctions removing the potential yield benefits from the applied strain.

From the results of this study we can draw conclusions about the mechanism that underlies sanctioning. It is now clear that the first step in the application of sanctions is for the plant to comprehensively detect and then integrate information about the global nitrogen status. This involves detecting the individual output of all existing nodules, this must be the case as we have shown that plants carry out sanctioning based on a global comparison. Given the variation in sanction severity based on the proportion of nodules occupied by a strain it must also be concluded that the plant mediates sanctions based on the number of nodules present, their fixation rates and the plants nitrogen requirements. In short, the plant assesses how many nodules it needs to invest in to meet its nitrogen requirements. Not only this but our results show that the comparison between nodule fixation rates occurs at an even finer resolution than previously shown. This remarkable detection and information integration then results in individual nodule sanctioning outcomes.

A potential mechanism therefore requires a global signalling system that outputs developmental changes in the root architecture based on nitrogen detection. Candidate signalling molecules therefore include the plant hormones, auxin, and abscisic acid (ABA). Auxin has been shown to play a crucial role in nitrate signalling (Vega et al., 2019) where nitrate modulates auxin transport and accumulation. This

process in part occurs via the action of the nitrate transceptor NRT1.1 (Krouk et al., 2010) which has already been hypothesised to play a regulatory role in sanctioning (Underwood et al., 2024). The hormone ABA is also a promising candidate for a signalling molecule in the application of sanctioning as it has been shown that nitrate stimulates ABA accumulation (Ondzighi-Assoume et al., 2016) while at the same time ABA plays a key role in in root architecture development (Signora et al., 2001). The role of ABA may be as a negative regulator of nodule sanctions as the presence of ABA inhibits expression of ABI1 which is a negative regulator of Snrk1 (Rodrigues et al., 2013) which is required for nodule malate production through the positive regulation of the genes MDH1 & 2 (Guo et al., 2023). This theory is further supported by the accumulation of ABA when nitrate is present (Ondzighi-Assoume et al., 2016) therefore it may be expected that ABA will accumulate around effectively fixing nodules.

This study demonstrates the extraordinary complexity of the regulatory mechanism of sanctioning as the plant must assess a myriad of factors to make individualised decisions for each nodule to maximise host benefit. Future studies must now look to elucidate the details of the genetic hormonal pathways that allow this incredibly intricate decision making to occur.

4.4 CONCLUSION

The work in this chapter tested three avenues for the evasion of sanctions by a 'cheating' strain. It was shown that local clustering of cheats was not a viable route to success, and it is inconclusive whether a smaller, but still significant, variation in fixation effectiveness may be undetectable to the sanctioning mechanism. However, this work did prove that if a cheater is able to dominate nodulation and therefore occupy a large majority of nodules then the pea has no choice but to reward this strain despite there being a more effective strain present. The evolutionary implications of this are fascinating. It would suggest that there is a strong selection pressure to improve nodulation amongst strains of lower fixation ability. This drive towards better nodulation however may be predicted to ultimately have no effect on symbiosis stability as amongst the members of a hyper nodulating strain there would then be a subsequent pressure for better fixation as amongst these hyper nodulating bacteria a better fixer may once again benefit from the improved treatment by the host-plant as well as the relative increase in fitness due to the punishment of their less effective cousins.

In demonstrating that sanctions are signalled globally, that they have a relatively fine resolution and that they may be attenuated based on the proportion of cheater nodules it is clear that the mechanism controlling sanctioning must allow for dynamic signalling, it must do so across the full root architecture, and it must be able to influence the development of nodules. These features led to the conclusion that within this signalling

system there could be a hormonal regulator. Our final goal therefore was to determine whether a plant hormone was controlling sanctioning.

5 Genetic and hormonal regulation of sanctioning

5.1 Introduction

To date, the focus of studies investigating sanctioning in the legume-*Rhizobium* symbiosis have been, almost universally, centred on the effects of sanctioning and demonstrating its evolutionary significance (Kiers et al., 2003; Regus et al., 2017; S. West et al., 2002; Westhoek et al., 2017, 2021). Very little work has been carried out investigating how sanctioning occurs. As a result, the mechanism behind sanctioning remains to be elucidated.

Having established in chapters 3 and 4 the physiological features of sanctioning, and a number of the factors that influence the severity of sanctioning, the final chapter in this thesis is focussed on the mechanism that regulates sanctions. The data collected in previous chapters had been used to infer and predict elements of this regulation. These findings suggested that it could be a plant hormone that acted as the signal to control sanctions.

To identify candidate regulators RNA sequencing and differential expression analysis of sanctioned and unsanctioned was carried out. The final list of genes of interest indicated that abscisic acid (ABA) could be a hormonal regulator. ABA addition assays, while not conclusive, did suggest that this could be the case.

Beyond the work on ABA as a candidate regulator for sanctioning this work also demonstrated the transcriptomic differences between a sanctioned and unsanctioned nodules. These lists of differentially expressed genes of interest provide a myriad of starting points for future work into the regulation of sanctioning.

5.2 ABA NEGATIVELY REGULATES THE APPLICATION OF HOST SANCTIONS IN THE LEGUME RHIZOBIA SYMBIOSIS

5.2.i Forward

The work in this chapter is presented in the form of a completed manuscript. This manuscript has not been submitted for publication as there is currently work underway as a part of a new doctoral student's project that will be combined with this work before its submission. The supporting information for this paper has been provided in Appendix C.

5.2.ii Abstract

Host sanctions are crucial for the evolutionary stability of the legume-*Rhizobium* symbiosis. While the physiological and evolutionary impacts of sanctioning have been well studied the mechanism of sanctioning is still uncharacterised. In this study we investigate this mechanism in the *Pisum sativum – Rhizobium leguminosarum* symbiosis. Transcriptomic analysis of sanctioned nodules revealed extensive transcriptional changes. In particular, a number of abscisic acid related genes were differentially expressed. This suggested a potentially regulatory role for the plant hormone in the regulation of sanctioning. This role was tested through hormone

addition assays in a split root system. These addition studies provided preliminary evidence that abscisic acid could recover the size of sanctioned nodules and did demonstrate that the hormone could recover fixation in a sanctioned nodule. This study improves our understanding of the transcriptomic effects of sanctioning and provides the first evidence for a candidate regulator of sanctioning.

5.2.iii Introduction

The symbiosis between leguminous plants and nitrogen-fixing bacteria allows them to meet their nitrogen requirements even in poor quality soils (Poole et al., 2018). This symbiosis is characterised by the exchange of carbon-based compounds, particularly C4-dicarboxylates, derived from photosynthesis for this fixed nitrogen (Udvardi & Poole, 2013). This exchange occurs within specialised root structures called nodules. In the presence of a compatible symbiont the legume forms these nodules to house the bacteria allowing them to replicate in a protective environment before being released back into the rhizosphere, at an elevated concentration, after nodule breakdown (Timmers et al., 2000). In this way engaging in the legume – rhizobia symbiosis provides a significant fitness benefit to the rhizobia.

Variation in the fixation effectiveness of wild rhizobia populations raises the possibility that a less effectively fixing strain may gain a fitness advantage by utilising the energy resources saved for replication. If this ‘cheating’ went unchecked it may lead to the breakdown of this symbiosis as the benefit to the host plant is eroded (R. F. Denison, 2000).

It has been demonstrated that to maintain the stability of this symbiosis, and to maximise the benefit to the host, legumes are able to punish less effectively fixing strains through nodule sanctions (Kiers et al., 2003; Oono et al., 2011; Westhoek et al., 2017, 2021). These sanctions have been shown to be conditional i.e. an intermediately effective strain may be sanctioned in the presence of a more effective strain but will be unsanctioned in the presence of a less effective strain (Underwood et al., 2024; Underwood & Poole, 2025; Westhoek et al., 2021). Conditional sanctions punish strains less effective at fixing N_2 by suspending the supply of carbon to these less effective nodules (Westhoek et al., 2021) as well as through the hastening of nodule senescence (Underwood et al., 2024).

While the effects of sanctioning on less effective strains, and on the nodules that house them, are increasingly well understood, there has been little work done on the underlying mechanism regulating how the host plant is able to discern between nodules of varying fixation and apply sanctions accordingly.

In this study we address this knowledge gap by using RNA sequencing of sanctioned and unsanctioned nodules to determine the transcriptional changes a nodule undergoes throughout sanctioning. By combining this transcriptomic understanding of sanctioning with the findings of previous studies on the control of sanctions several candidate genes for key regulatory functions in this mechanism were identified. As a number of these genes were related to the plant hormone abscisic acid (ABA) it was determined that ABA was a strong candidate for a key regulatory role in sanctioning.

Hormone addition experiments provided further evidence for this role while leaving a number of unanswered questions as to the exact mode of action.

Our findings provide a first look into the extraordinarily complex regulatory mechanism that controls sanctioning in the legume-*Rhizobium* symbiosis. The list of differentially expressed genes in sanctioned versus unsanctioned nodules offers numerous avenues for future studies to follow. In this study we investigated the role of the plant hormone ABA and based on our findings we propose that it plays a regulatory role in host sanctioning.

5.2.iv Methods

Bacterial strains and growth

Both of the strains used in this work were derived from *Rhizobium leguminosarum* bv. (Rlv) 3841. A root symbiont of *Pisum sativum* and a highly effective fixer of atmospheric nitrogen (Johnston & Beringer, 1975). The strains used were a Fix^+ strain, the wild-type Rlv 3841, and a Fix^{int} strain which fixes at approximately 50% the capacity of the Fix^+ strain (Westhoek et al., 2021). These strains are near isogenic and varied only in fluorescent reporter tag, to distinguish between nodules of either strain, and the region upstream of the *nif*A promoter where the Fix^{int} strain had an Ω spectinomycin cassette insertion which throttled the strains fixation effectiveness (Table 1). Strains were maintained on tryptone-yeast agar (TY) agar (Beringer, 1974) with the required antibiotics (Table 1). For longer term storage strains were inoculated into a solution of TY with 15-20% glycerol and stored at -80 ^oC. For inoculation of peas,

strains were grown on TY agar slopes. Slopes were washed with UMS (Poole et al., 1994) and the concentration of cells was calculated by measuring the OD600 using a Genesys 250 UV-Visible spectrophotometer. This solution was then diluted to approximately 1 x 10^7 cells per mililitre.

Plant growth

For RNA sample extraction *Pisum sativum* seeds from the genome sequenced cultivar JI2822 (Rayner et al., 2024) were used. For ABA addition experiments the cultivar Avola was used as this has been the model for previous conditional sanctioning studies (Underwood et al., 2024; Westhoek et al., 2017, 2021). Seeds were sterilised by washing with 70% ethanol for 1 minute followed by washing with 20% bleach (NaClO) for five minutes. Seeds were then rinsed with sterile water to remove residual ethanol and/or bleach. Following sterilisation seeds were germinated on 1% w/v agar plates in the dark at 28^{o}C for five days.

Following five days of germination the roots of all peas were cut laterally, removing the tip of the primary, with the cut placed just above the point where root hairs had begun to develop. These cut peas were placed on 1% w/v agar plates which were sealed round their edges with micropore tape and placed in a growth chamber (21 °C, 16 h photoperiod) for five days.

After five days, once substantial lateral roots had formed from the sides of the cut primary root, half of the lateral roots were placed into a 500 ml Azlon beaker containing a 1:1 mix of silver sand and fine vermiculite. The other half of the roots were placed

into a second identical beaker. To each of these beakers 75 ml of nitrogen free rooting solution (Westhoek et al., 2021) was added along with 5 x10^6 cells of the desired strain of *R. leguminosarum*. The combination of strains was either a Fix^+ strain in one beaker and a Fix^{int} strain in the second or Fix^{int} was applied to both beakers. To prevent contamination cling film was applied over the beakers with a slit cut to allow the pea shoot to grow through. The pots were then placed in a growth chamber (21 °C, 16 h photoperiod).

RNA extraction

After 10 or 17 days post inoculation (dpi) peas were harvested and the two root sections removed from their respective pots. Using a sterile scalpel all nodules from a root section were removed, placed in Eppendorf tubes, and immediately flash frozen in liquid nitrogen. Samples were collected from both sides of three plants of each strain combination (Fix^+ & Fix^{int} or Fix^{int} & Fix^{int}) for both time points. RNA extraction was carried out using the Qiagen RNAeasy Plant Mini Kit.

RNA quantity and quality was assessed using the Agilent 2100 Bioanalyzer system for samples of RNA for each nodule type: Fix^+ (co-inoculated with Fix^{int}), Fix^{int} (co-inoculated with Fix^+) and Fix^{int} (co-inoculated with Fix^{int}). These three nodule types are henceforth referred to as Fix^+, Fix^{int} (sanctioned) and Fix^{int} (unsanctioned). Three samples for each nodule type which met the minimum requirements for sequencing by Novogene (100 ng of RNA in a volume of no less than 10 µl and at a concentration of no less than 10 ng/µl. For RNA quality the minimum value of RNA integrity number (RIN) was 4.0).

Genome alignment

Novogene provided, primer sequence trimmed, RNA sequencing files for each of the samples. These reads were aligned using Bowtie 2 (Langmead & Salzberg, 2012) to a Bowtie 2 index for the RefSeq *P. sativum* annotated genome (Yang et al., 2022) available from NCBI (O'Leary et al., 2016) built using the Bowtie 2 build function. Alignment output a sequence alignment map file (SAM) and a log text file of alignment statistics. For all but one sample the percentage alignment exceeded 97%. The sample that failed to do so (B2) aligned at only 13.78%. However, this still resulted in $7.26x10^6$ aligned reads for this sample. While this was approximately ten-fold fewer than the other samples (Table S1) it still provides ample coverage for the pea genome with over a hundred and fifty times more reads than the total gene coding regions in the genome.

Feature counting

Counting the number of aligned reads mapping to each of the gene coding regions of the genome required the SAM formatted output files of Bowtie 2 converting to sorted binary alignment map (BAM) format files. This was done using the samtools programme (Li et al., 2009). To generate a count for each annotated gene the programme htseq count assigned the mapped reads to the corresponding annotated gene based on chromosome and base pair position provided in the general transfer format (GTF) file published along with the genome by NCBI.

Differential Expression analysis

To compare the relative number of reads for each gene was compared between each of the three nodule types to find genes that were significantly differentially expressed using DEseq2 (Love et al., 2014) on the Galaxy.eu server (Afgan et al., 2018). The list of significantly differentially expressed genes were annotated with the assigned function for the gene provided in the GTF file using DESeq2 annotation on the Galaxy.eu server.

Functional group annotation

Genes of interest were assigned as genes that were significantly differentially expressed when comparing Fix^{int} (sanctioned) with Fix^{int} (unsanctioned) and/or Fix^{int} sanctioned vs Fix^{+} and were not significantly differentially expressed when comparing Fix^{int} (unsanctioned) with Fix^{+}. The list of genes interest was annotated into functional groups based on the gene's protein record in Uniprot (Bateman et al., 2025).

Exogenous ABA addition

To test the effect of ABA on nodules split roots were inoculated with Fix^{+} and Fix^{int} strains in separate pots. At 7 dpi an ABA solution was added to either the Fix^{+}, the Fix^{int} or neither pot of the split roots. 50 ml of either a 0.5 μM (for the nodule measurement experiment) or 0.05μM (for fixation effectiveness experiment) ABA solution was applied. At 28 dpi plants were harvested and removed from the pots. At this point either the five largest nodules from each side of each plant, based on visual

inspection, were photographed and measured using the Fiji image analysis software (v2.14.0/1.54f), as in Underwood & Poole (2025), or the two halves of the root system were assessed for fixation effectiveness through an acetylene reduction assay.

Acetylene reduction assay

The acetylene reduction assay was carried out as described in Westhoek et al., (2021) with some adjustments for the split root protocol. Rather than whole plants being placed into each Schott bottle, the two root sections for each of the split root plants were separated from the other half of the roots as well as from the shoot. These root sections were then placed into individual Schott bottles.

Statistical analysis

The size or fixation rate of Fix^{+} or Fix^{int} nodules was compared across treatments through a one-way ANOVA and then if a significant difference was found the treated nodules were compared to the control treatment through a Dunnett's post-hoc test.

Table 1: Rhizobial strains

All strains derived from Rlv3841 and provided with a strain code, resistance markers, short description and reference.

Name	Strain	Antibiotic resistance	Description	Reference
Fix^{+} GFP	OPS1339	Streptomycin, Gentamicin	Rlv3841 Tn7-Gm- GFP	(Westhoek et al., 2021)
Fix^{int} mCherry	OPS2269	Streptomycin, Spectinomycin, Gentamicin,	Fixint Tn7-Gm- mCherry (Rlv3841 mutant, ΩSpc cassette in promoter region of *nifA*)	(Westhoek et al., 2021)

5.2.v Results

Sanctioned and unsanctioned nodules show large variability in transcriptome

In the 10 dpi samples there were 152 genes of interest identified that were significantly differentially expressed when comparing a sanctioned to an unsanctioned nodule (Table S2) of which 38 were without a gene name annotation. In the 17 dpi samples there were 360 genes of interest identified (Table S3) of which 97 were without a gene name annotation. The two sets of genes had little overlap with only eight genes appearing in both and fourteen genes with the same gene annotation appearing in both (Fig. 1). The annotated genes differentially expressed in the 10 and 17 dpi samples were grouped based on function. In the set of 10 dpi genes of interest there were seven distinct functional groups which each contained more than five percent of the characterised genes of interest (Table 2). In the set of 17 dpi genes there were ten distinct functional groups containing 5% or more of the characterised genes of interest (Table 2).

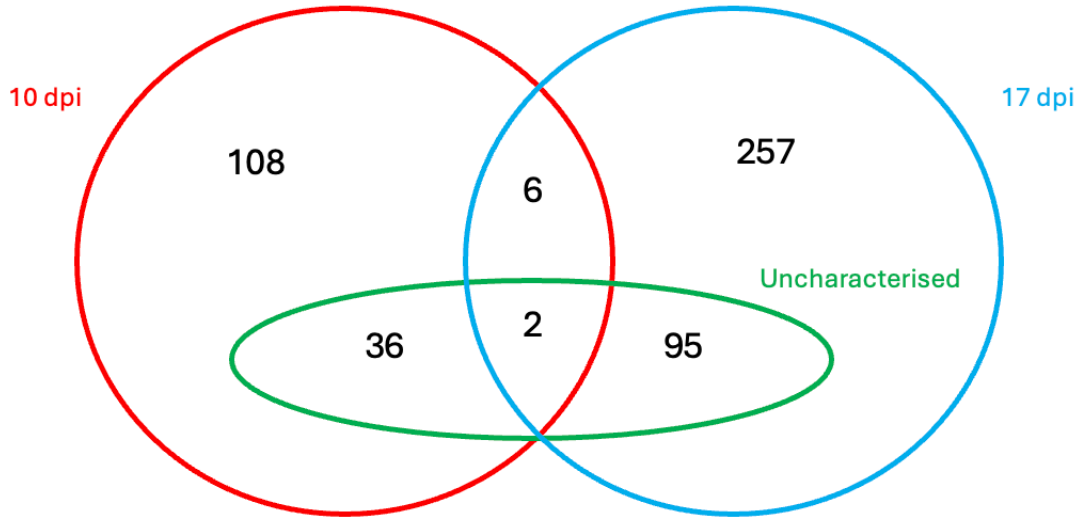

Fig 1. The transcriptomes of sanctioned nodules at 10 and 17 dpi show little overlap

The significantly differentially expressed genes in a sanctioned vs unsanctioned nodule were compared between nodules harvested 10 and 17 days post inoculation. Based on the unique locus tag for each gene the two lists were compared to find how many genes appeared in both lists. Some genes had not been designated a functional annotation and are therefore classified as uncharacterised. The number of uncharacterised genes in each group, and overlapping between groups, was also counted.

Table 2: Functional groups within genes of interest

Functional groups assigned to genes differentially expressed in sanctioned compared to unsanctioned nodules at either 10 or 17 days post inoculation (dpi). Groups containing 5% or more of genes reported.

Functional group	10 dpi (% of genes)	17 dpi (% of genes)
Hormone regulation	✓ (16%)	✓ (9%)
Metabolism	✓ (15%)	✓ (12%)
Stress Response	✓ (19%)	✓ (15%)
Cell wall	✓ (6%)	✓ (5%)
Defence	✓ (6%)	✓ (8%)
Disease	✓ (9%)	
Post-transcriptional regulation	✓ (6%)	
Transport		✓ (14%)
Development		✓ (9%)
Programmed cell death		✓ (6%)
Post-translational regulation		✓ (6%)
Symbiosis		✓ (5%)

ABA related genes are downregulated in sanctioned nodules

Three ABA responsive genes were significantly downregulated in the 10 dpi RNA samples from sanctioned compared to unsanctioned nodules. There were also two WRKY 40 transcription factors, shown to negatively regulate ABA signalling (Geilen & Böhmer, 2015), significantly up regulated in sanctioned nodules compared with unsanctioned nodules (Table 3). While several hormone related genes were differentially expressed in the samples only the ABA related genes showed a consistent regulatory pattern.

Table 3: Differentially expressed ABA related genes

Genes significantly differentially expressed in a sanctioned versus an unsanctioned nodule. Gene name is the RefSeq gene id for the gene, Annotation is the RefSeq annotation provided in the genome, Log_2 fold-change is relative to the unsanctioned control and p-value has been adjusted for multiple comparisons by the programme DESeq2.

Gene name	Annotation	Log_2 fold change	p-value
LOC127073242	ABA-responsive protein ABR18-like	-2.908	0.03684328
LOC127073246	ABA-responsive protein ABR18-like	-7.044	6.0514E-05
LOC127073248	ABA-responsive protein ABR18-like	-3.573	0.0030659
LOC127091022	probable WRKY transcription factor 40	3.892	0.0450314
LOC127100887	probable WRKY transcription factor 40	1.385	0.02942466

ABA addition may recover nodule size in sanctioned nodules

To test for a role of ABA in sanctioning the hormone was added exogenously to either side of the split root system. If, as predicted based on the differential analysis, ABA was a negative regulator of sanctioning it was hypothesised that when ABA was added to the sanctioned (Fix^{int}) side of the split root then the sanctioned nodules would recover in size in comparison to the control.

Application of ABA to the Fix^{+} side of a split root system resulted in an increase in the size of the Fix^{int} nodules on the same plant, when compared to the Fix^{int} nodules from the untreated control plants (Fig. 2). However, this increase in size was not statistically significant. Unexpectedly, there was no change in size, compared with the untreated control, for the Fix^{int} nodules in the split root systems where ABA was applied to the Fix^{int} side. Fix^{+} nodules did not vary in size compared with the untreated control plants in the plants treated with ABA on either side. The increase in size of Fix^{int} while not significant, was consistent with the hypothesis of ABA as a regulator of sanctioning.

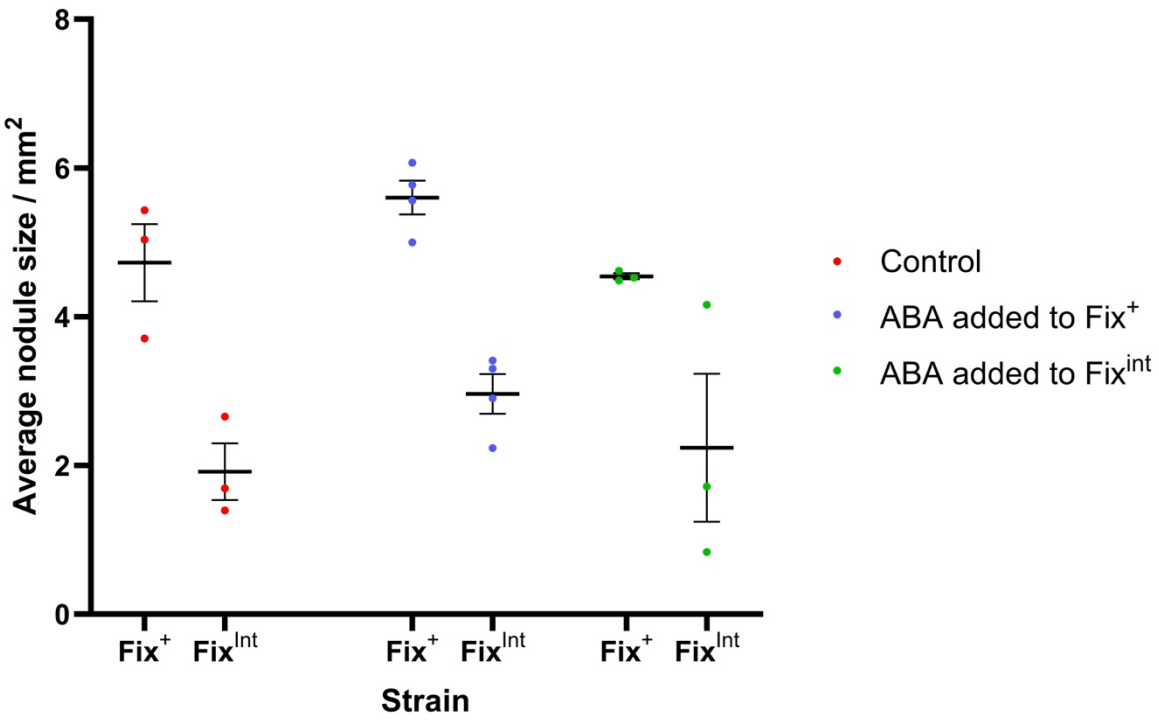

Fig. 2: ABA addition partially recovers the size of sanctioned nodules

Split root pea plants spread across two pots were inoculated with a Fix^+ and Fix^{int} strain into the separate pots. After seven days plants were given one of three treatments: a control treatment of water to both pots, 50 ml of 0.5 µM ABA to the Fix^+ pot, or 50 ml of 0.5 µM ABA to the Fix^{int} pot. Plants were harvested after 28 days and the average nodule size measured. Black bars indicate mean values. Coloured points indicate individual plant means, error bars indicate the standard error of the mean.

ABA addition recovers fixation in sanctioned nodules

It has been previously shown that sanctioned nodules do not fix a significant amount of nitrogen even though the strains within the nodules are capable of fixation. Therefore, it was hypothesised that should ABA be a negative regulator of sanctioning then applying ABA should restore fixation in a sanctioned nodule. To test this, split roots were treated with ABA as in the previous experiments this time with a lower concentration (0.05μM) and the root sections were tested for fixation.

In the Fix^{int} nodules that were opposite a Fix^{+} pot which had been treated with ABA there was a significant level of fixation. This was significantly higher than the level of fixation in control sanctioned Fix^{int} nodules (estimate = 23.003, 95% CI = 11.769 : 34.237, p = 0.0003). However, when testing the Fix^{int} nodules that had been directly applied with ABA there was no fixation. For the Fix^{+} pots it was seen that regardless of treatment the Fix^{+} nodules continued fixing. However, in the case of the Fix^{+} nodules opposite a Fix^{int} pot treated with ABA there was a significant increase in the level of fixation (estimate = 68.771, 95% CI = 17.345 : 120.196, p = 0.0101) (Fig. 3).

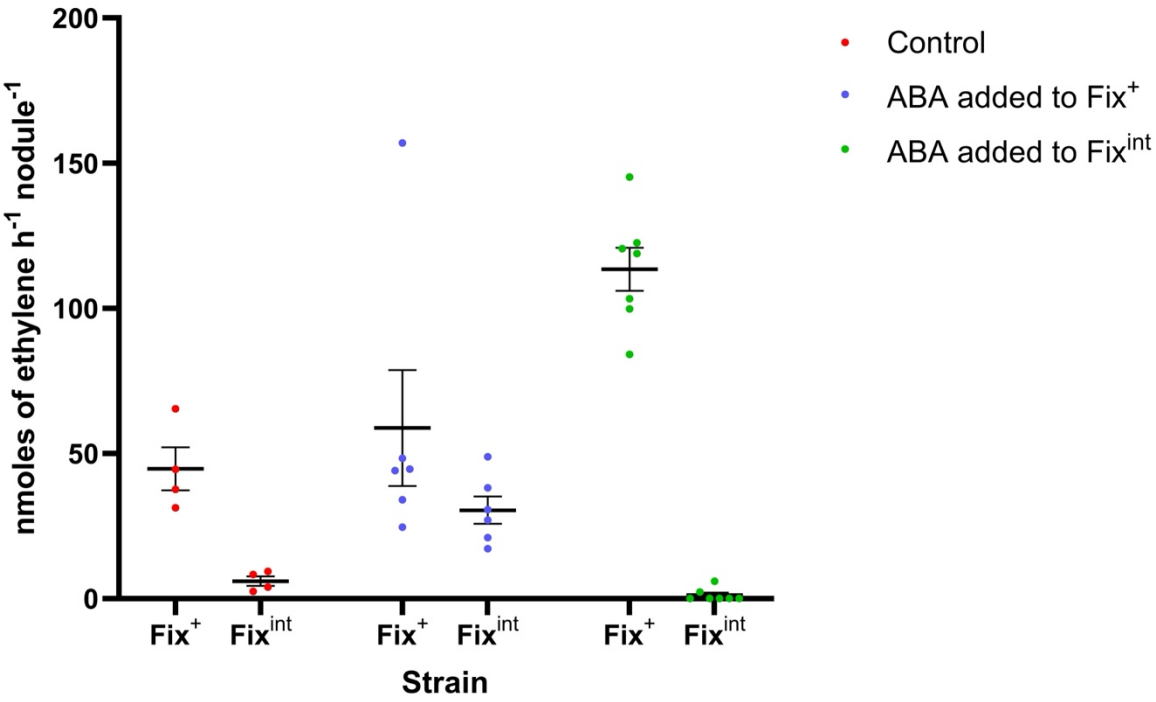

Fig. 3: ABA addition partially recovers fixation in sanctioned nodules

Split root pea plants spread across two pots were inoculated with a Fix^+ and Fix^{int} strain into the separate pots. After seven days plants were given one of three treatments: a control treatment of water to both pots, 50 ml of 0.05 µM ABA to the Fix^+ pot, or 50 ml of 0.05 µM ABA to the Fix^{int} pot. Plants were harvested after 28 days and the fixation rate was measured through an acetylene reduction assay. Black bars indicate mean values. Coloured points indicate individual plant values, error bars indicate the standard error of the mean.

5.2.vi Discussion

In this manuscript we have investigated the transcriptional changes that occur in a sanctioned nodule at two different time points. Surprisingly, the 10 dpi RNA samples already had a clear indication that sanctioning was occurring. There was a large number of metabolism genes differentially expressed in the sanctioned nodule including sugar transport protein 13 which was down-regulated (log2 fold change -1.548).

This was a surprising result as it doesn't align with the findings of Westhoek et al., (2021) in which it was shown through luminescence reporter constructs that the transport of sucrose to a sanctioned nodule had not dropped, relative to an unsanctioned nodule, as of 12 dpi. However, there was a significant difference seen after 20 dpi.

At the same time within the sanctioned nodules there was already significant differential expression of genes relating to defence and stress response. This clearly indicates that the active process of signalling and applying sanctions has already begun as of 10 dpi. Therefore, while we had predicted that our 10 dpi samples would provide a view into the nodule transcriptome before sanctioning these results demonstrate that in fact these samples appear to show the transcriptome at a very early stage of sanctioning.

This conclusion is supported by the differential expression of the LOB domain-containing protein 37-like, involved in nitrogen metabolism (Majer & Hochholdinger, 2011) which was also down-regulated (log_2 fold change -2.381). Thereby demonstrating that the nitrogen status of the sanctioned nodule was already compromised relative to the unsanctioned nodule as of 10 dpi. Overall, it suggests that the transcriptional luminescent reporters used by Westhoek et al., (2021) have a significant delay in their response to sugar levels relative to RNAseq analysis of transcription.

For our 17 dpi RNA samples the genes differentially expressed did not appear to be related to the signalling and detection around nitrogen fixation but instead seemed to relate to the active application of sanctions. The clearest example of this would be the differential expression of the leghaemoglobin genes as this is a molecule essential for proper nodular function and the provision of oxygen to the bacteria in a hypoxic environment. At 17 dpi there was also a large number of genes relating to the cell wall and to programmed cell death.

This was intriguing as previous work (Underwood et al., 2024) has shown that one of the effects of sanctioning is the premature breakdown of the cells within a sanctioned nodule. However, this has been shown to occur only after 35 dpi. In fact, after 28 dpi the cells within a sanctioned nodule still appear healthy. Therefore, as in the case of carbon metabolism for the 10 dpi data, this is further evidence for the transcriptome of a sanctioned nodule pre-figuring physiological changes that will be observed at a later time point.

As our results indicate that the decision making behind sanctioning occurs at an earlier time point than previously expected it was concluded that the 10 dpi genes of interest were more likely to include candidate regulators such as VAPRYN-like-like and CASP-like.

VAPRYN -like-like

The VAPRYN gene, -1.395 log_2 FC down regulated in sanctioned nodules at 10 dpi, is essential for both the symbiosis between land plants and arbuscular mycorrhizal funghi (AMF) (Pumplin et al., 2010) and the legume-*Rhizobium* symbiosis (Murray et al., 2011). It is known that VAPRYIN is required for correct initiation and growth of infection threads (Liu et al., 2019). Potentially the change in VAPRYIN expression prevents further development of existing infection threads to punish the undifferentiated bacteria within these sanctioned nodules.

CASP-like protein

CASP-like proteins (-3.24 log_2 FC down-regulated in sanctioned nodules) (CASPLs) are a group of transmembrane proteins known to localise to the casparian strip membrane domain. While it is known that nodules do contain a casparian strip the function of the casparian strip in this context is not yet fully understood (Hartmann et al., 2002). However, it has been shown that the casparian strip is involved in metabolite homeostasis (Shen et al., 2025). This suggests that the casparian strip, and CASPLs, plays a key role in controlling the flow of metabolites into a nodule. By

restricting the flow of metabolites, the downregulation of CASPLs may contribute to sanctioning.

Within this gene list there were also a number of hormone related genes. Therefore, it was clear that hormones were a strong candidate signalling molecule, especially given the well understood function of many hormones to control a variety of developmental processes. Abscisic acid was a particularly promising candidate given the clear expression pattern within ABA related genes pointing to ABA as a candidate for negative regulation of sanctioning i.e. in the presence of ABA a nodule is not sanctioned.

The application of ABA to our split root system provided preliminary evidence for the plant hormone playing a key role in sanctioning. The failure to recover nodule size or fixation in the Fix^{int} nodules directly applied with ABA, when coupled with the partial rescue of nodule size and the successful rescuing of fixation when the Fix^{+} nodules were applied with ABA, suggests that ABA may be capable of abolishing sanctions, but only when applied distally.

This may be because the abolishment of sanctioning requires the transport of ABA into the nodule. When ABA is applied to a section of root the uptake of ABA may be expected to raise the concentration of ABA in that section. This raise in concentration may induce a concentration gradient between the different sections of the root. Therefore, ABA may move across the root architecture out of and away from the area of application and into the un-treated region of roots. If the effect of ABA was then dependant on the triggering of ABA channels moving into the nodule this would result

in the lessening of sanctions for the nodules that are physically separate from the site of ABA application but not for those where the ABA was applied. This theory is consistent with the current literature which has shown that the transport of ABA into a tissue is crucial for the hormones mechanism of action (Kaur et al., 2025) and that ABA may be transported across the plant through simple diffusion because it is a weak acid (Boursiac et al., 2013).

Alternatively, it has also been shown that the activity of the ABA biosynthesis gene *ABA2* within phloem companion cells may increase the levels of ABA in a site specific manner (Mehra et al., 2022) i.e. *ABA2* activity may be specifically elevated in the companion cells adjacent to a more effective nodule. In this way the absolute quantity of ABA within a nodule may be altered. This hypothesis would, similarly to the global transport hypothesis, require linking the output of the nodule to the expression of a *ABA2* but would offer a simpler theory as to how ABA levels may be raised in a site specific manner.

These hypotheses are supported by the known function of ABA as it relates to nodule malate supply. ABA is known to inhibit the gene ABI1 (Rodrigues et al., 2013)which itself inhibits SnRK's. At the same time SnRK1 is known to drive MDH 1 & 2 which promote malate production in the nodule (Guo et al., 2023). To confirm this potential role for ABA in sanctioning more work must be carried out at a wider range of concentrations as well as in variations of split root set up to test the effect of distally applying ABA.

In conclusion, this study has shown that the transcriptional impacts of sanctioning begin earlier than previously supposed and that the transcriptome of a sanctioned nodule is significantly different by just 10 dpi. We have also demonstrated that the plant hormone ABA, which is differentially expressed in a sanctioned nodule, can abolish some features of sanctioning. This is theorised to occur either via the transport of ABA into a sanctioned nodule or through elevated ABA levels mimicking increased ABA biosynthesis. However, this result requires validation to prove this negative regulatory function for ABA.

This validation could be achieved using an ABA biosynthesis mutant such as the *wilty* mutant of *P. sativum* (McAdam et al., 2015). In this mutant the lack of ABA would, if our hypothesis is correct and ABA is a negative regulator of sanctioning, result in sanctioning of all nodules regardless of the fixation effectiveness of their occupant.

5.3 CONCLUSION

My aim in this chapter was to utilise the improved understanding of sanctioning developed in earlier sections of this thesis in order to investigate the mechanism regulating sanctioning. RNA sequencing and hormone addition results have provided new insights into this mechanism. The identification of ABA as a candidate regulator of sanctioning is a major step forward in our understanding of the mechanism controlling sanctioning. At the same time these results have opened up an array of new questions for researchers to begin working on.

A key outcome of this chapter and this thesis is the list of differentially expressed genes for sanctioned versus unsanctioned nodules. Theoretically these lists of genes for 10 and 17 dpi contain the key genetic regulators of sanctioning. Several of these genes were particularly promising candidates for future study.

The addition of ABA while not conclusive did appear to support the theory that ABA could be a regulator of sanctioning. There were however a number of other hormone related genes showing significant differential expression.

A number of Auxin related genes were differentially expressed in the 17 dpi dataset. This included a probable indole-3-acetic acid-amido synthetase, a negative regulator of auxin activity (Peat et al., 2012) and three WAT1-related proteins. WAT1 regulates auxin homeostasis within plant cells (Ranocha et al., 2011). Unlike the ABA related genes at 10 dpi there was not a clear pattern in terms of negative or positive regulation of auxin. However, the clear dysregulation of auxin homeostasis does suggest a role for auxin in sanctioning.

While this chapter was focussed on hormonal regulation, because hormone addition studies were achievable within the timeline available, the non-hormone genes of interest also contain a number of promising candidate genes for further study. These examples demonstrate that the work in this chapter has yielded numerous new avenues for research particularly for plant geneticists.

A road-block in this study was the lack of bioinformatic resources for *P. sativum.* In particular, the quality of gene annotation for the genome was relatively poor. The

genome of the cultivar selected for sequencing (JI2822) currently has all genes annotated as ‘uncharacterised’ and even the best annotated genome, the Reference genome provided on NCBI of the Zhongwan6 cultivar, still contains a large number of “as yet uncharacterised genes”. As described in the manuscript the list of genes of interest contained 38 and 97 genes for 10 and 17 dpi respectively. These uncharacterised genes of interest may be essential genes for the proper signalling and application of sanctioning. Therefore, as work on the pea genome continues and hopefully the annotations of these genomes improve this sequencing data should be returned to and re-annotated to account for newly described genes.

The results of this chapter demonstrate that sanctioning is the result of a complex genetic and hormonal regulatory network. While the key regulators of this network are yet to be elucidated it is very likely that they are to be found within the genes of interest provided in this manuscript such as VAPRYINs & CASPLs. In conclusion this work provides fresh insight into the regulation of sanctioning and provides a number of avenues for future research.

6 Discussion

6.1 SUMMARY OF RESULTS

6.1.i Chapter 3

The major results of this chapter were: A) That the application of conditional sanctions by the legume on a less effective or 'cheating' strain result in a radical change within the nodule. Thereby going beyond the whole nodule morphological effects shown in previous work such as Westhoek et al., (2017) & (2021). Here it was shown that sanctions result in a significant decrease in the number of nitrogen-fixing bacteroids but not of the non-fixing undifferentiated bacteria by 28dpi. After 35dpi however there was clear changes to the internal cell structure of nodules containing a less effective strain suggesting that sanctioning results in premature senescence of less effective nodules. B) That within a mixed nodule the host plant (at least in the case of *Pisum sativum*) is incapable of differentiating between the two strains present within the same nodule. This was shown by the equal treatment of the two strains despite the variation in fixation effectiveness.

6.1.ii Chapter 4

The focus of chapter four was understanding how less effective nitrogen-fixing bacteria were still prevalent in wild populations despite the effect of conditional sanctions in reducing their fitness. It was shown that strains may evade sanctions if they dominate nodulation, occupying a majority of nodules, but that spatial separation from a better fixer had no impact on sanctioning severity. I was unable to show

conclusively whether or not a small variation in fixation may allow a less effective fixer to evade sanctions. However, I did show that a variation smaller than those tested in previous studies could still be accurately perceived by the host plant.

6.1.iii Chapter 5

In chapter five I established that there were significant transcriptomic differences between sanctioned and unsanctioned nodules as early as ten days post-inoculation. These changes were across several functional groups but particularly in genes relating to hormonal regulation. The effect of one of these hormones, ABA, when applied to the side opposite sanctioned nodules, is the recovery of fixation in the sanctioned nodules. From this it was concluded that ABA plays a key role in regulating sanctioning.

6.2 General discussion

The overall aim of this thesis was to improve our understanding of sanctioning as a mechanism for host-control in the legume – *Rhizobium* symbiosis. In doing so the results from this thesis provide a starting point to discuss the implications across a variety of fields including the evolution and evolutionary stability of symbioses like this one.

6.2.i Evolutionary consequences

One theme of this study has been an effort to understand the nature of this symbiosis. In some circles a symbiosis is still viewed as a purely mutualistic interaction in which both partners come together to exchange a good and or service that the other needs so that both may benefit (Leigh, 2010). A more cynical view might be that in the exchange the partners are providing something of little value to themselves in return for something they are in much greater need of. In either scenario however this may be viewed as a ‘win-win’ scenario.

However, given the laws of natural selection, in which the only goal is to boost individual fitness, can an individual ever engage in such an exchange without a pressure to cheat their partner and reduce, or eliminate entirely, its offering in the exchange (Archetti et al., 2011). Regardless of context, participation in a symbiosis incurs an energetic, and therefore fitness, cost. Given this, it could be argued that all symbioses should eventually, either collapse into parasitism, as one partner finds a way to extract what it needs for free, or be abandoned by both partners as the race to parasitise each other ends with no exchange what-so-ever.

There are cases of individual sacrifice. An organism ‘choosing’ to lower their own fitness in favour of boosting the fitness of another such as in bee, ant or termite colonies (Sun et al., 2018). However, while phylogenetically very distinct, these are all eusocial animals (Shimoji & Dobata, 2022) and therefore any ‘sacrifice’ they are seen to make is in fact covered by Hamilton’s rule (Hamilton, 1964a, 1964b). Hamilton’s rule, In short, states that when a bee sacrifices its own fitness for the good of its hive

it is in fact protecting its the fitness of its own genes as other members of the hive or future offspring of the queen are genetically identical. This, obviously, cannot be the case for an example such as the legume-*Rhizobium* symbiosis. In this study we have shown that this symbiosis is kept stable not due to the mutualistic actions of both but due to the punitive efforts on the part of the legume to keep cheaters in check and enforce fair play.

We might therefore hypothesise that in stable symbioses the partners may be engaged in an evenly matched evolutionary arm wrestle in which neither side can gain an advantage. Thereby giving the appearance of a stable position. This is similar to the Red-Queen hypothesis (Van Valen, 1973) which states that when two species co-evolve, they must undergo constant change and adaptation in order to maintain stability between the two. When applied to the example of the legume-*Rhizobium* symbiosis this would imply that the nitrogen-fixing bacteria are continually evolving and adapting in a continual push for improved fitness. One avenue for this improved fitness being to 'cheat' and fix less nitrogen but receive the same benefit from the legume (R. F. Denison, 2000). At the same time the legume host is also continually adapting to effectively punishing cheating bacteria in order to maximise its nitrogen supply and fitness. In doing so this ever-escalating arms race will appear to be a stable symbiosis.

While it is true that Hamilton's rule cannot apply to rhizobia and their legume hosts, it is worth considering how a cheating individual may impact the other members of its population. Given that unchecked cheating will, in the long run, result in the loss of the

symbiosis, cheating rhizobia are in fact detrimental to their cooperative relatives. Therefore, not cheating could be viewed altruistically, by not benefitting from the apparent fitness benefits of cheating a rhizobium may boost the fitness of the other members of the population.

Another consideration when thinking about the impact of cheating is that the fitness cooperative rhizobia and their hosts are both negatively impacted by cheating. Taking this idea one step further it means that effective sanctioning by the legume host is actually a fitness benefit to non-cheating rhizobia.

Sanctions do not, however, guarantee the long-term stability of the symbiosis. It may be the case that eventually one partner will gain the upper hand, e.g. a rhizobium will emerge that is able to evade sanctions while cheating, effectively turning the mutualism into a parasitism. This is far from inevitable though as there are examples of more ancient symbioses persisting across evolutionary time. The most notable of these is the plant - mycorrhizal funghi symbiosis, which is present in more than 80% of land plants (Martin & van der Heijden, 2024) and believed to have emerged 450 million years ago (Delaux & Gutjahr, 2024), nearly 400 million years before the emergence of the legume – *Rhizobium* symbiosis (De La Peña et al., 2018). An example of how a mutualism between partners who cannot be behaving according to Hamilton's rule therefore may remain stable.

The pressure to cheat, and as a logical extension, the collapse of the symbiosis that may follow the emergence of successful cheating may explain the paraphyletic distribution of the rhizobial symbiosis amongst the member of the legumes, and the

rosids more broadly (Doyle, 2016). It is currently a widely debated topic as to whether this distribution has arisen via convergent evolution in multiple individuals (Kates et al., 2024) or through a single gain followed by sporadic loss of the symbiosis (Griesmann et al., 2018). Given that it has now been shown how a cheating bacterium may evade sanctions, through dominating nodulation, there is a potential route for the loss of the symbiosis in individuals due to the dominance of cheats. This result therefore provides some support for the multiple loss theory of the evolutionary history of this symbiosis. The complexity of the sanctioning mechanism as outlined in this thesis also works against the theory of multiple emergences. Following Occam's razor, the simplest explanation would be that this incredibly intricate system emerged once and has then been lost multiple times, as loss may require a single mutation but gain requires assembling a complex regulatory network.

Understanding how evolution has shaped this symbiosis and how it may or may not remain stable is particularly important given the agricultural significance of the legume-Rhizobium symbiosis and the agricultural goals of those who work on it.

6.2.ii Agricultural consequences

Sanctioning is a crucial factor when considering the use of the legume-*Rhizobium* symbiosis in agriculture. Firstly, when using rhizobia to inoculate a field of leguminous crops with the intention to use fixation of atmospheric nitrogen to boost the nitrogen content of the soil. Effective sanctioning is critical if the benefit of this inoculation is to be maximised.

In chapter four of this thesis, it was demonstrated that sanctioning may only work effectively to reduce the fitness of a less effective fixing strain when the majority of nodules are occupied by the more effective strain. If a majority of nodules are occupied by a less effective strain, the level of energy expended by the plant to achieve the same nitrogen uptake will be higher than when a majority of nodules are occupied by a more effective strain. This increase in energy expenditure could otherwise have been used for additional growth and yield improvement.

To ensure that rhizobial inoculants result in the best yield possible the other strains already present in the soil and their relative nodulation rates and fixation effectiveness must be considered (Triplett & Sadowsky, 1992). If a strain of great nodulation capacity and lower fixation effectiveness is present in the soil then the benefit from the use of an inoculum will be limited at best.

A second area where sanctioning must be integrated into current thinking is the engineering of nitrogen fixation into cereal crops. The generation of a cereal crop capable of engaging in the legume-*Rhizobium* symbiosis and benefitting from the fixed nitrogen would result in a dramatic reduction in the use of artificial fertilisers (Oldroyd & Dixon, 2014; Rosenblueth et al., 2018). Thereby also reducing the associated economic cost to farmers and the inherent instability in price of fertilisers, as well as the environmental cost of fertiliser use and fertiliser production. However, the benefits of these currently hypothetical crops are contingent on the symbiosis generated being effective and stable. Therefore, during the process of engineering these crops it is critical to also engineer into these ‘artificial’ symbioses the ability of the host-plant to

sanction ineffectively fixing strains. Without doing this there would be a strong selection pressure on the nitrogen-fixing bacteria to reduce or even eliminate the rate of nitrogen fixation.

Finally, sanctioning is an essential component to the maintenance of a healthy symbiosis within the plant species that already engage in it. Therefore, the results of this thesis suggest that work on leguminous crop plants should also take this into account. This can be expressed most clearly when considering breeding programmes for the agriculturally important legumes such as soybean or peanut. When modern breeding programmes are assessing the ability of new cultivars it could be argued that effective sanctioning is a trait that must be bred for alongside maximising yield or stress tolerance.

For these agricultural goals to be achieved the first step must be developing a fundamental understanding of mechanisms that regulates sanctioning.

6.2.iii Proposed mechanistic model

The results of this thesis have demonstrated that the mechanism behind sanctioning requires a global comparison of nitrogen output at the nodule level. At the same time integrating information about the relative frequency of the more or less effective nodules. This work has also demonstrated that this occurs at a remarkably early time point (prior to 10 dpi) and is modulated through the absence of the plant hormone abscisic acid. A mechanistic model of how sanctioning occurs and the consequences to the nodule over the course of nodule development may now be constructed. This is

achieved by integrating what is already known about the symbiosis, my results, and hypothesising on likely systems.

0 – 5 dpi

During this initial phase of infection, rhizobia are drawn by chemotaxis towards the root surface where they are able to attach and begin colonising (Wheatley et al., 2020). There will be an exchange of chemical signals triggering a signalling cascade eventually resulting in root hair curling and infection thread formation. The bacteria divide down the infection thread and begin to occupy the cells of the newly developing nodule (Poole et al., 2018).

5 – 10 dpi

Once the nodule has formed the bacteria are exposed to NCR peptides (Montiel et al., 2017). These peptides trigger the terminal differentiation into nitrogen-fixing bacteroids. The bacteroids begin to fix nitrogen which is then transported out of the nodule. I would suggest that the transportation of this fixed nitrogen must in some way trigger a signalling mechanism that is proportional to the amount of nitrogen transported. One theory for this may be the action of a nitrogen transceptor (Krouk et al., 2010; Zhang et al., 2019), proteins capable of acting as a receptor and a transporter for nitrogenous compounds.

This detection of nodule output could then trigger the ABA accumulation in the more effective nodule. As this accumulation must be dependent on the output of the other nodules it seems likely that there would be an intermediate step between the detection

of nitrogen output and the accumulation of ABA. For example, the nitrogen signalling could drive the recruitment of channel or transporter proteins for ABA (Boursiac et al., 2013; Kaur et al., 2025). This could then drive the movement of ABA at a level proportional to the amount of nitrogen being exported. Assuming the total quantity of ABA spread through the root system is finite then the flux of ABA would always be towards the best fixing nodules and these nodules would accumulate a majority of the ABA. This theoretical model based on ABA flux would then account for conditional sanctioning (Fig. 1A).

This model would also account for the proportional effect seen in chapter four. When better nodules are scarce the total ABA accumulated into these nodules would be lower allowing for more ABA to accumulate into the less effective nodules.

Alternatively, the detection of nodule output may drive the activity of local ABA biosynthesis, potentially through the activity of *ABA2* within local phloem companion cells (Mehra et al., 2022). In this system it would be the accumulation of malate in better nodules around the root system, rather than ABA, that would allow for conditional and proportional sanctioning (Fig. 1B).

Either system could then drive sanctioning by the change in malate accumulation caused by the change in ABA levels. As has been discussed previously in this thesis ABA can drive expression of SnRK1 which is known to drive nodule malate production (Rodrigues et al., 2013).

However, neither malate dehydrogenase or SnRK's were shown to be differentially expressed in our samples. This could have been caused by a variety of factors, most notably the large number of uncharacterised genes within the pea genome. If the genes involved are particularly nodule specific homology based annotation with a better annotated genome e.g. *Arabidopsis thaliana,* would not have annotated these genes correctly if at all.

By 10 dpi the nodule nitrate output could then be detected and the associated changes in ABA levels may be affected.

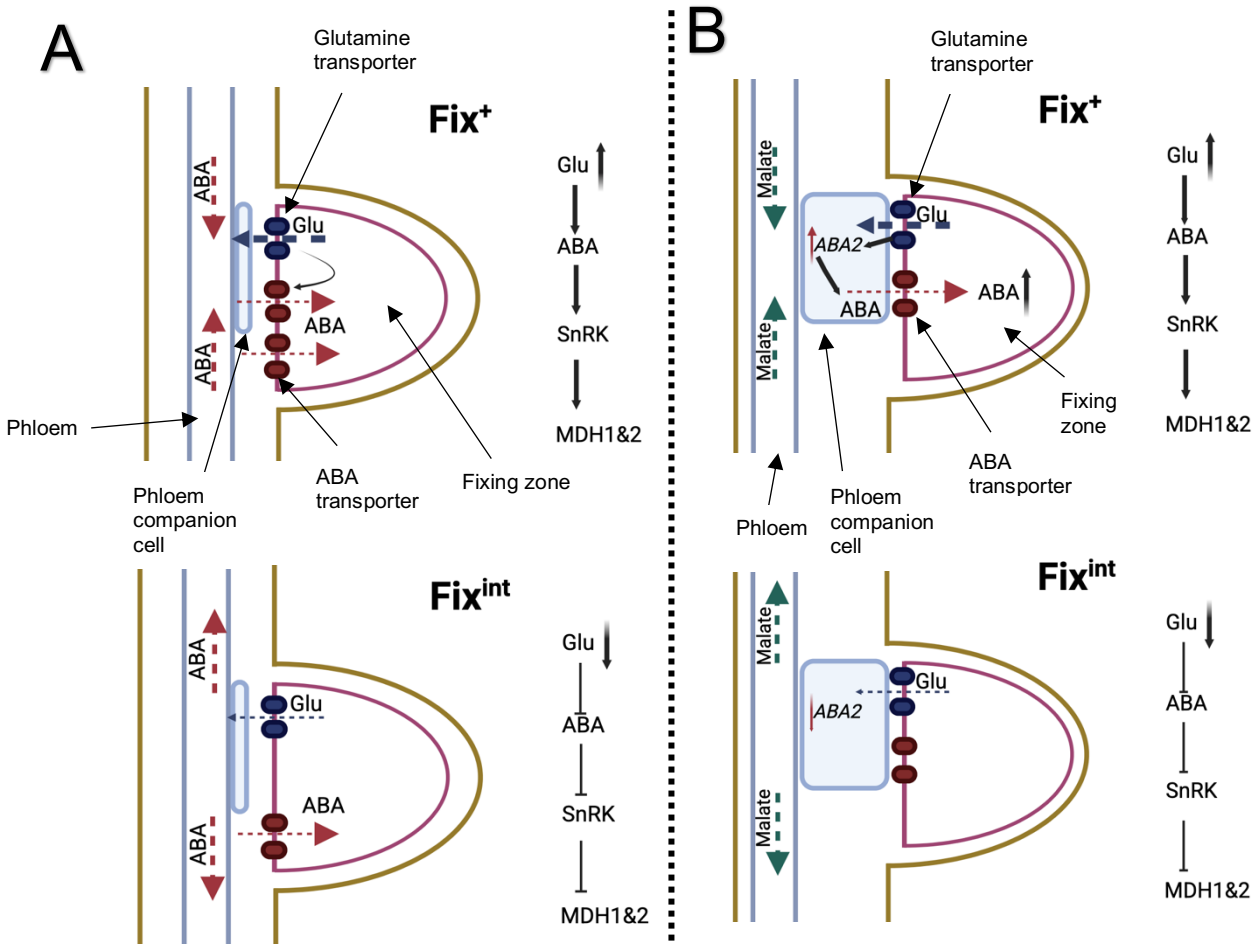

Fig. 1 Models for ABA mediated sanctioning

In the first model (A) ABA flux mediates the application of sanctioning. In this model the transport of fixed nitrogen as glutamine leads to increased activity of ABA transporters. This increases the ABA flux towards more effective nodules and away from less effective nodules. The relative flux of ABA towards more effective nodules allows this model to account for sanctioning that is conditional on the relative effectiveness of the other nodules on the plant. In the second model (B) the transport of glutamine increases the activity of the ABA biosynthesis gene *ABA2* within the phloem companion cells. This increases ABA levels within more effective nodules, e.g. a Fix^{+} nodule, whereas in less effective nodules, e.g. a Fix^{int} nodule, less ABA is accumulated. In this second model the increased flux of sugars, in the form of malate, rather than ABA flux makes the model conditional on the effectiveness of other nodules. In both models the increased glutamine transport leads to the accumulation of ABA in the more effective nodule. The increased ABA levels drive the increased activity of SnRK's which increase the activity of malate dehydrogenase 1 & 2.

10 – 20 dpi

Following this hypothetical change in ABA levels, as well as the associated change in carbon supply, more dramatic transcriptional effects will begin to occur. By 17 dpi a number of developmental genes have been differentially expressed resulting in arrested development a reduction in size for sanctioned nodules (Westhoek et al., 2017). Genes relating to programmed cell death have also been up regulated which begins the process of nodule senescence however this will not come into visual effect for some time. The carbon levels within the sanctioned nodule also begin to change more dramatically at this point as by 20 dpi the carbon supply to a sanctioned nodule has effectively stopped (Westhoek et al., 2021).

20 – 28 dpi

Now that the carbon supply has stopped the bacteroids within the sanctioned nodules are starved of energy and begin to die off leading to a collapse in the number of bacteroids within a sanctioned nodule. The bacteria surviving within the infection threads become saprophytic at this point (Timmers et al., 2000) and metabolise the remains of the now dead bacteroids. This process may allow the number of bacteria within a sanctioned nodule to increase.

28 – 35 dpi

The programmed cell death genes, up-regulated after 17 dpi, result in visible effects as the cells within the sanctioned nodule undergo apoptosis, losing their spherical shape and rupturing. This premature senescence combined with the total loss of

carbon supply to the nodule will result in the starvation of the remaining bacteria and the eventual collapse of their numbers (Westhoek et al., 2021).

35 dpi +

Nodule senescence continues as in an unsanctioned nodule with the breakdown of the remaining plant cells. Eventually the nodule will completely break down and a limited number of bacteria will be released back into the rhizosphere, significantly fewer then are released from an unsanctioned nodule.

6.3 Future Directions

6.3.i Testing the sanctioning regulatory mechanism

The next step in the progression from this work would be the testing of the regulatory model proposed in this thesis. Having proposed that the hormone ABA is a key factor in the regulation of sanctioning the first step following on from this thesis must be to test the relative levels of ABA in sanctioned and unsanctioned nodules. Hormone relative abundance studies have been carried out on a variety of plant tissues including quantification of ABA in plant tissue. These protocols would allow measurements of ABA levels at individual time points which would enable the confirmation of my results indicating a drop in the ABA levels within sanctioned nodules.

Live imaging of hormones within plant tissues using system such as FRET (Forster resonance energy transfer) based fluorescent imaging would also be required to visualise the transport of ABA around the root system of the plant and could be used to confirm the mode of action behind the results for the ABA addition assay. FRET

based imaging works using two fluorophores which will only both fluoresce when in close proximity (< 10 nm) to each other. In the case of ABA studies researchers have tagged two proteins which interact in the presence of ABA with the two fluorophores (Balcerowicz et al., 2021). Therefore, by tracking fluorescence it is possible to track the movement and accumulation of ABA throughout a plant.

One of the key outputs of this thesis is the transcriptome data for sanctioned nodules. This data set of genes of interest is an excellent start point for any future studies into the genetic regulation of sanctioning. A reverse genetics screen focussed on creating knock-out mutants for some or all of the genes of interest and examining the effect on the host-plants ability to sanction effectively would be one avenue. This could be achieved by moving away from *P. sativum* thereby negating the lack of bioinformatic tools available for pea. The most obvious candidate for a host legume would be *Medicago truncatula* as it is widely studied and has a large number of genetic tools available (Ye et al., 2025). A flaw with this work scheme is the lack of an intermediate fixing strain that is capable of infecting *M. truncatula* making the generation of an intermediate fixing strain of *Sinorhizobium meliloti* 1021 an essential first step.

While the bioinformatic tools for pea are not as plentiful as for other systems, there are a number of genetic tools that may be exploited. one example is the Pisum germplasm collection held by the John Innes centre. By using a core diversity panel from this collection there is large degree of variation across the genome throughout this panel which have been sequenced for their variation from the ZW6 reference genome (Yang et al., 2022). Therefore, by screening individuals in this panel, with

mutations in already identified genes of interest, for defects in sanctioning it may be possible to rapidly isolate regulatory genes. Alternatively, as we have already identified ABA as a candidate regulator, the screening of the well characterised ABA biosynthesis mutant, *wilty* (McAdam et al., 2015), would test this regulatory role directly.

A different approach that would utilise the genes of interest list generated in this thesis would be to instead take a forward genetics approach. This may be achieved using a *P. sativum* TILLING population generated through random mutagenesis (Dalmais et al., 2008). Or this could be achieved more effectively in *M. truncatula* using a Tnt1 insertion mutant library (Tadege et al., 2008). In the Tnt1 mutant library each line contains, on average, twenty-five randomly inserted transposons. This population would then be screened for loss of sanctioning. The specific transposon, of the twenty-five in each line, responsible for the phenotype is found by assessing the gene annotation for the genes each transposon is inserted within; this is where the list of already identified genes of interest would accelerate the study. These genes responsible can then be confirmed by targeted mutagenesis e.g. using the CRISPR Cas9 system. The draw-back of the Tnt1 population compared with the *P. sativum* TILLING population is that it would again necessitate the construction of a new intermediate fixing strain.

Regardless of model system, any forward genetics approach would be a large-scale experiment to undertake. To use the Tnt1 population as an example; the *M. truncatula*

genome would require screening approximately 15,000 lines to achieve 90% coverage of the genome (Tadege et al., 2008).

6.3.ii Construction of novel intermediate fixers

To date only two, isogenic, intermediate fixing strains have been generated. These two strains are the *R. leguminosarum* 3841 Fix^{int} & Fix^{L} strains used in this thesis. Intermediate fixing strains are essential for any experiment seeking to understand sanctioning. Several studies have compared the treatment of Fix^{+} and Fix^{-} isogenic strains across a number of model systems (Chen et al., 2024; Daubech et al., 2017; Oono et al., 2011; Westhoek et al., 2017). However, the treatment of Fix^{-} strains is fundamentally different to the treatment of a less effective strain. When a non-fixing strain occupies a nodule this nodule will never begin to fix nitrogen whereas a less effective strain will begin fixing initially. As such a non-fixing strain could be shut down much earlier as well as being treated more akin to a pathogen then a symbiote. It is now well documented that plants encounter strains of varying fixation in wild populations (Berrada et al., 2019; Woliy et al., 2019) and therefore only strains of varying fixation should be used to simulate these dynamics. What is more the use of isogenic strains is also critical as this is the only way to ensure there is no competition or fitness impact on the strain beyond its treatment by the plant (R. Denison, 2021). When non isogenic strains are used in studies it becomes increasingly difficult to separate the characteristics of a strain due to its genetics and the impacts of sanctioning.

The generation of novel intermediate fixing strains would allow for a comprehensive study of sanctioning across the legumes and more distantly related nodulating plants. A study of this nature would be able to confirm the presence of conditional sanctioning beyond *P. sativum* as well as providing important insights into the evolution of sanctioning in this symbiosis. Moving away from *P. sativum* would also, as discussed above, open up the range of molecular and genetic tools available for future studies.

There are a number of possible approaches to constructing novel intermediate fixing strains. To discuss these in a practical context the example of *S. meliloti* 1021 will be used. One approach would be to simply replicate the two intermediate fixing strains already produced either by inserting an antibiotic resistance cassette into the upstream region of *nif*A or by knocking out *fix*L. Taking these in turn, insertion upstream of *nif*A is far from guaranteed to produce a Fix^{int} strain. The original Fix^{int} mutant was produced while attempting to produce a *fix*C mutant but the cassette was inserted between *nif*A and *fix*X instead leading to disrupted read through from the autoregulating NifA dependent promoter upstream (Westhoek et al., 2021). A fresh insertion may not result in the same impact on NifA autoregulation and could have no impact or even produce a Fix^{-} strain.

As for the knock-out of *fix*L a different problem emerges, the variation in genetics between nitrogen-fixing bacteria. While *R. leguminosarum* 3841 carries two copies of fixL (one on the chromosome and one on the symbiotic plasmid) (Rutten et al., 2021). *S. meliloti* carries only one (on the symbiotic plasmid) (Geddes et al., 2021). The Fix^{L} strain used in this thesis was a double knock-out of both copies (Rutten et al., 2021).

Knocking out the single copy of fixL in *S. meliloti* may not have the same impact on fixation as in *R. leguminosarum*.

While these potential issues do not prevent these methods being attempted in *S. meliloti*, when scaled across the diversity of bacteria needed to carry out a representative study across the legumes it becomes clear that a more consistently effective method is required.

An alternative approach that may produce more consistent results may be to construct a simple biological circuit with a variety of ribosome binding sites to modulate expression levels driven by the endogenous promoter. For example, this may be done for the expression of *nif*H, an essential gene for nitrogenase construction (Barloy-Hubler et al., 2000), modulated with a variety of ribosome binding sites and inserted into the chromosome through a mini-tn7 system. If done in a *nifH* knock-out mutant the insertion of these constructs should yield mutants showing a range of fixation effectiveness. Crucially this method should be transferable across the range of rhizobia as *nif*H is an essential element in nitrogen fixation (Westhoek et al., 2017).

One weakness in the use of lab generated intermediate fixers in the study of sanctioning is that they only provide insight into one very specific form of cheating. While limiting fixation is perhaps the most obvious form of cheating and comes with the clearest fitness benefit there may well be other mechanisms by which a bacterium may benefit from the symbiosis without contributing. For example, while mechanistically challenging to achieve, if a strain was able to piggyback with a highly effective strain into a mixed nodule but fail to then exit the infection thread. Then by

staying in an undifferentiated form the rate of fixation by the nodule would remain high while this 'cheat' could benefit from the supply of malate and the protective environment of the nodule. In this example the strain would not see the same level of fitness benefit as the cooperative strain but it would have an advantage over strains that remain in the rhizosphere while going undetected based on our current understanding of the application of sanctions. Future work will eventually have to consider these other potential mechanisms of cheating.

6.4 CONCLUSION

The results of this thesis demonstrate that the legume-Rhizobium symbiosis is not maintained by an inherently cooperative exchange, but by a complex and dynamic system of host-imposed sanctions that constrain the fitness of less effective rhizobia. By integrating evolutionary theory, physiological data and transcriptomic evidence this work shows that sanctioning occurs at an early stage within nodule development and through a systemic regulatory process. This process is dependent on hormone signalling and relative nodule performance. These results also show that sanctioning can explain the stability of the symbiosis. As well as how, in some conditions, sanctioning fails, potentially leading to the loss of the symbiosis. The implications of this work when applied to agriculture highlight that sanctioning is not simply a theoretical construct but instead has real-world implications for the pursuit of food security while limiting the use of artificial fertilisers. Ultimately, this thesis proposes that the apparent stability of the legume–*Rhizobium* partnership is best understood as

a finely balanced evolutionary arms race, continually renegotiated at the molecular, ecological, and evolutionary levels.

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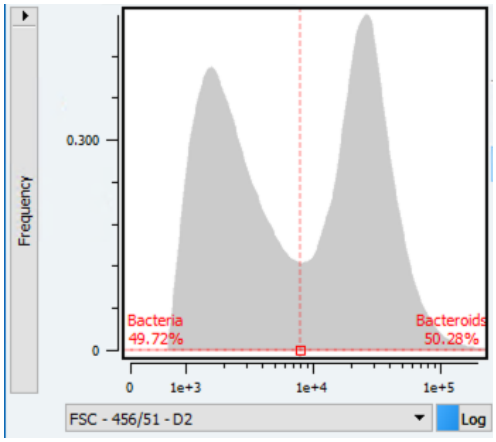
Appendix A

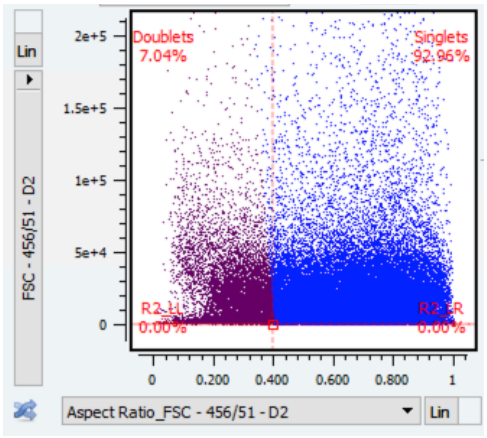
The following Supporting Information is available for Chapter 3:

Fig. S1 Nodules were picked 28 days post inoculation, crushed, and passed through a flow cytometer. The events were gated based on size to separate out bacteria and bacteroid events (A). There are two clear populations of different within a nodule. The larger population are the bacteroids. The gating line is shown in red at approximately 8000 arbitrary units FSC. Events were also gated based on aspect ratio to identify singlets and doubles (B). Gating line is at 0.400 FSC aspect ratio. To identify bacteria gating of fluorescence was used as bacteria were fluorescently tagged with mCherry or GFP. GFP bacteria were gated at values above 4000 arbitrary units (C). Nodules containing mCherry tagged bacteria showed negligible values for GFP fluorescent events (D). Bacteria tagged with mCherry were gated at values above 6000 arbitrary units (E). Nodules containing GFP tagged bacteria showed negligible values for GFP fluorescent events (F).

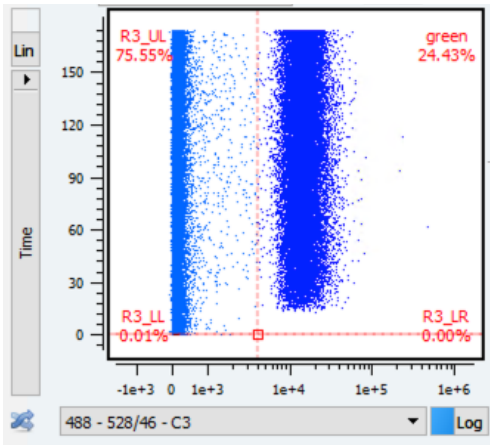
A


Frequency
0.300
0
Bacteria
49.72%
Bacteroids
50.28%
0
1e+3
1e+4
1e+5
FSC - 456/51 - D2
Log

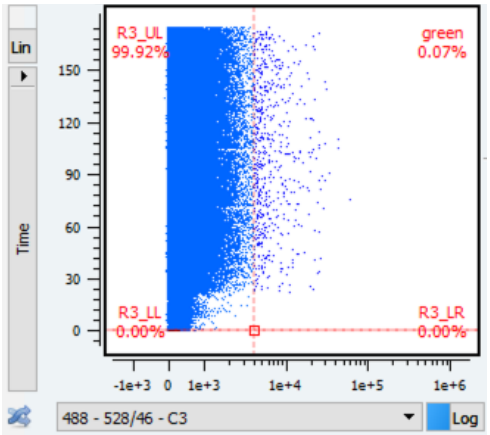
B


Lin
FSC - 456/51 - D2
2e+5
1.5e+5
1e+5
5e+4
0
Doublets
7.04%
Singlets
92.96%
R2_LL
0.00%
R2_LR
0.00%
0
0.200
0.400
0.600
0.800
1
Aspect Ratio_FSC - 456/51 - D2
Lin

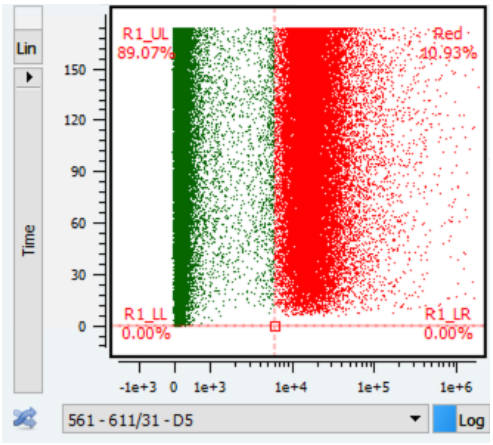
C


Lin
Time
150
120
90
60
30
0
R3_UL
75.55%
green
24.43%
R3_LL
0.01%
R3_LR
0.00%
-1e+3
0
1e+3
1e+4
1e+5
1e+6
488 - 528/46 - C3
Log

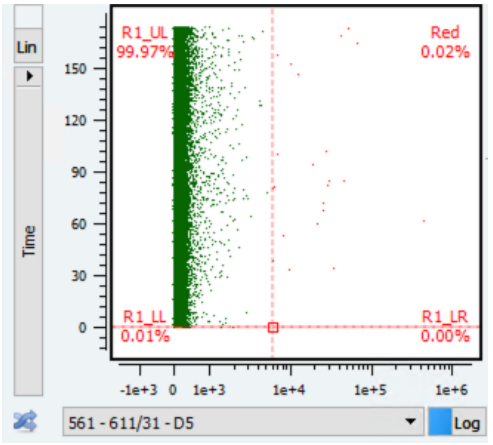
D


Lin
Time
150
120
90
60
30
0
R3_UL
99.92%
green
0.07%
R3_LL
0.00%
R3_LR
0.00%
-1e+3
0
1e+3
1e+4
1e+5
1e+6
488 - 528/46 - C3
Log

E



F



Doc. S2 Flow cytometry data, analyzed using custom gating to calculate the number of undifferentiated bacteria and bacteroids in single occupancy and mixed nodules, and acetylene reduction assay and nodulation results from fluorescent tag control experiments. This data is available from the BioRxiv pre print server doi: https://doi.org/10.1101/2024.04.25.582971

Doc.S3 Flow cytometry data was analyzed in R using R studio. Code for analysis is provided in the form of an R markdown file. This file is available from the BioRxiv pre-print server doi: https://doi.org/10.1101/2024.04.25.582971

Fig. S4 Formulae for back transformation for outputs of analyzes of $\log_{10}$ transformed data

$$E = 10^{A} - 10^{(A + B)}$$

$$SE = (10^{A} - 10^{(A + B)}) - (10^{A} - 10^{(A + B + C)})$$

Where:

E is the back transformed estimate difference between the two means

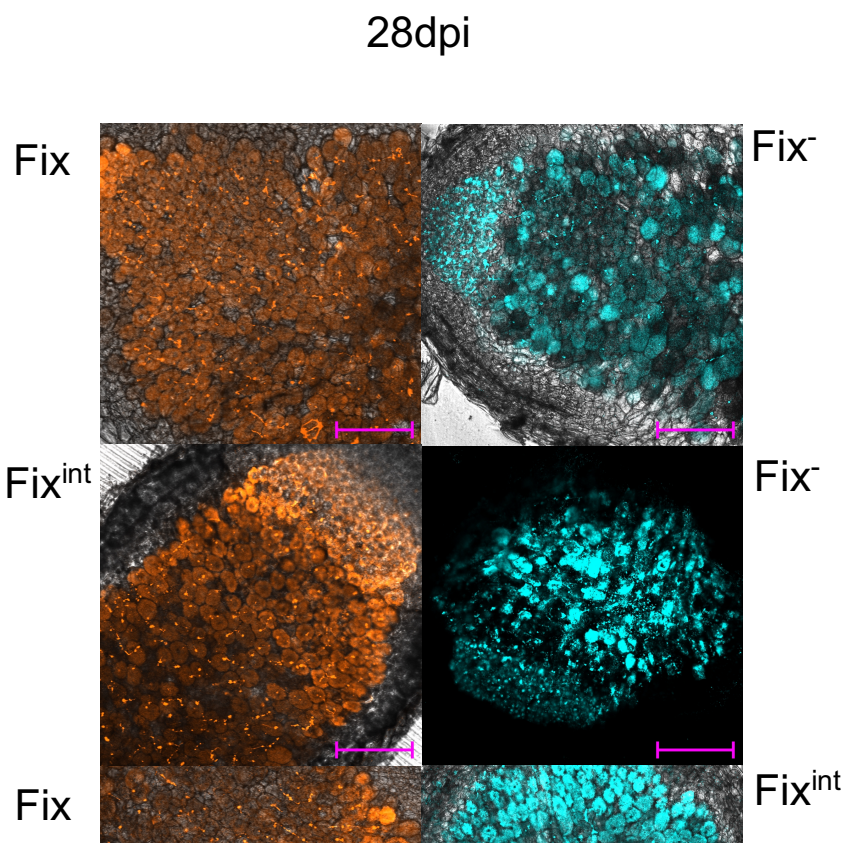
A is the $\log_{10}$ transformed estimate of the mean of the focal population

B is the estimate difference between A and the $\log_{10}$ transformed estimate of the comparison population

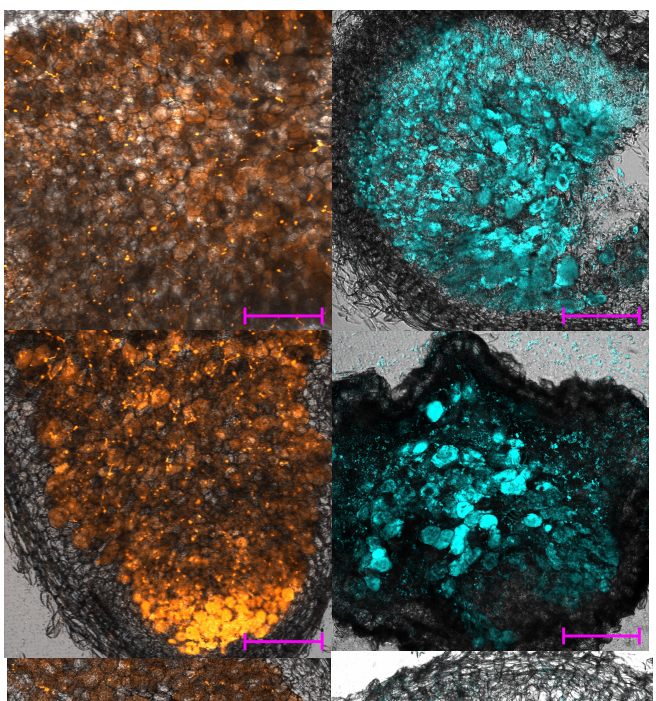
SE is the back transformed standard error of the estimate difference between the two $\log_{10}$ transformed means

Fig. S5 Confocal images of single occupant nodule sections: Peas were inoculated with one of three combinations of strains: Fix^{+} & Fix^{-}, Fix^{int} & Fix^{-} and Fix^{+} & Fix^{int}. Strains were isogenic apart from fluorescent tag and fixation ability. 100µm slices were taken from nodules picked after 28, 35 and 42 days post inoculation for imaging. Images were assessed for evidence of cell death. After 28 days there was limited evidence for a change to cell health between the nodules containing the effective

(Orange) and the ineffective (Turquoise) strains. After 35 days all nodules containing the effective strain remained healthy while the ineffective containing nodules showed evidence of cell death. After 42 days all nodules regardless of occupant showed clear evidence of cell death.



35dpi



42dpi

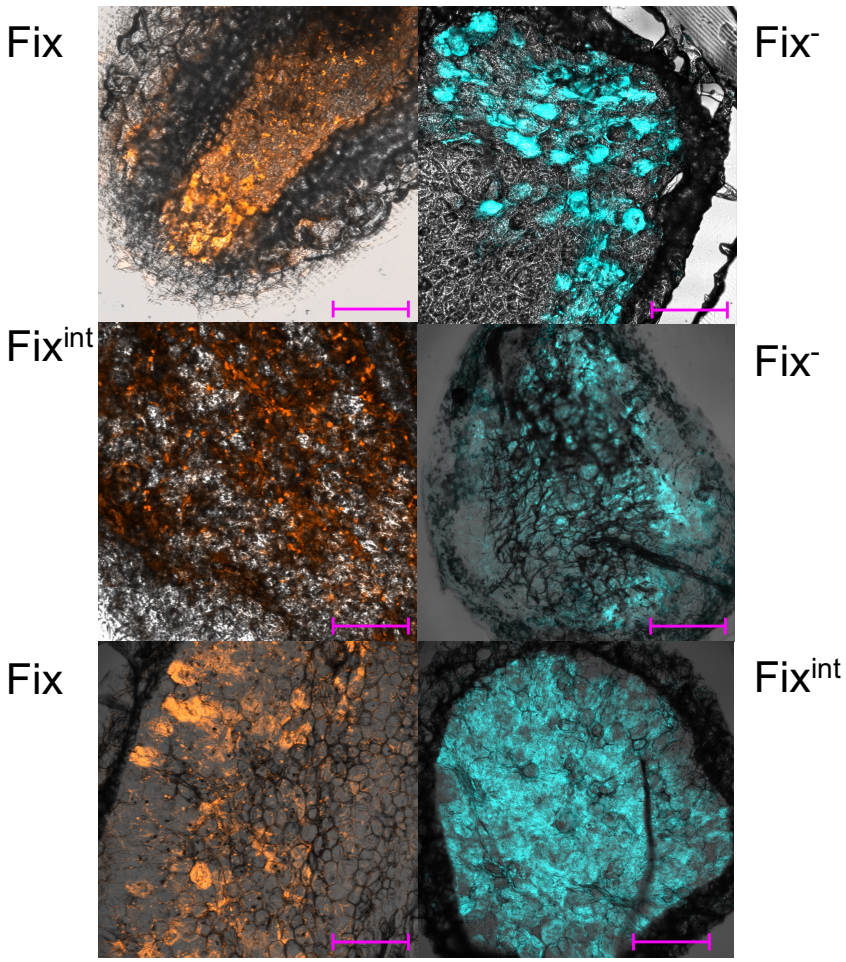


Fig. S6 Analysis of the number of bacteria of each strain within a mixed nodule

Fig. S7 Analysis of the number of bacteria within a mixed nodule

Values from mixed-effects models on the difference in the number of bacteroids between the two strains within a mixed nodule. Data that has been Log_{10} transformed is indicated by (log_{10}). P-values exceeding 0.05 are reported as >0.05.

Mixed nodule combination	Estimate	Standard error	t	p	n
Fix^+ & Fix^-	2.48 x 10^5 (log_{10})	48390 (log_{10})	3.675	0.0015	21

Fig. S7 Analysis of the number of bacteria within a mixed nodule compared to the single nodule

Fig. S8: Analysis of the number of bacteria in mixed and single occupant nodules

Values from mixed-effects models on the difference in the number of bacteria within a mixed nodule and the more (A) or less (B) effective single nodule. Data that has been log_{10} transformed is indicated by (log_{10}). P-values exceeding 0.05 are reported as >0.05.

A

B

Mixed nodule vs less effective single nodule	Estimate	Standard error	t	p
Fix^+ & Fix^- vs Fix^-	$-6.54x10^6$ (log_{10})	$3.65x310^6$ (log_{10})	7.914	<0.001

Appendix B

The following supporting information is available for chapter 4:

Mixed nodule vs more effective single nodule	Estimate	Standard error	t	p
Fix^+ & Fix^- vs Fix^+	$3.63x10^5$ (log_{10})	$5.83x10^5(log_{10})$	0.918	>0.05

File S1: RMarkdown file for statistical analysis of the data collected for the manuscript.

The file is available for download from the BioRxiv preprint server doi: https://doi.org/10.1101/2025.08.21.671492

Table S2: Data for nodule size and acetylene reduction assay for all experiments in this manuscript. Data is available for download from the BioRxiv preprint server doi: https://doi.org/10.1101/2025.08.21.671492

Appendix C

The following supporting information is available for chapter 5:

Table S1: Alignment statistics for RNA samples

Sample	Harvest time point	Nodule occupant (co-incoulant)	Overall alignment %	aligned reads
A1	10 dpi	Fix^{+} (Fix^{int})	99.45	5.03E+07
A2	10 dpi	Fix^{+} (Fix^{int})	99.42	5.38E+07
A3	10 dpi	Fix^{+} (Fix^{int})	99.27	6.86E+07
B1	10 dpi	Fix^{int} (Fix^{+})	97.19	5.70E+07
B2	10 dpi	Fix^{int} (Fix^{+})	13.78	7.26E+06
B3	10 dpi	Fix^{int} (Fix^{+})	98	6.68E+07
C1	10 dpi	Fix^{int} (Fix^{int})	99.09	5.30E+07
C2	10 dpi	Fix^{int} (Fix^{int})	99.21	6.06E+07
C3	10 dpi	Fix^{int} (Fix^{int})	99.33	5.33E+07
D1	17 dpi	Fix^{+} (Fix^{int})	99.27	5.91E+07
D2	17 dpi	Fix^{+} (Fix^{int})	99.36	5.34E+07
D3	17 dpi	Fix^{+} (Fix^{int})	99.08	5.64E+07
E1	17 dpi	Fix^{int} (Fix^{+})	98.58	7.11E+07
E2	17 dpi	Fix^{int} (Fix^{+})	99.42	6.82E+07
E3	17 dpi	Fix^{int} (Fix^{+})	99.3	5.88E+07
F1	17 dpi	Fix^{int} (Fix^{int})	99.28	4.98E+07
F2	17 dpi	Fix^{int} (Fix^{int})	99.24	5.69E+07
F3	17 dpi	Fix^{int} (Fix^{int})	99.54	5.76E+07

Table S2 & S3: Differentially expressed genes of interest for 10 and 17 dpi

Data is available for download from Zenodo.org

https://zenodo.org/uploads/17200918?token=eyJhbGciOiJIUzUxMiJ9.eyJpZCI6IjM4OGQ3ZDg1LWYzN2QtNGE2Ny05NTgxLTc0NTBmMzUzMTQ5OCIsImRhdGEiOnt9LCJyYW5kb20iOiIxNTA4NGJmMjM3M2ZjNjBhMGJlZTNkNWZhZDE1NjYwNiJ9.Rl2DhywS252OUE3VLHevmULxlTDu69BeDdmdNwC8gGsungOUlzxD3JCUfc2z81DEIY2_lBED9Gs3VIH07lytOw