LEGUME RHIZOBIUM SCIENCES

18th Australian Nitrogen Fixation Conference 2022

ANFC2022

5th - 8th December 2022

Conference Program and Abstracts

Murdoch University

GRDC GRAINS RESEARCH & DEVELOPMENT CORPORATION

GOVERNMENT OF WESTERN AUSTRALIA
Department of Primary Industries and Regional Development

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Foreword

It is with great pleasure that we welcome you to the 18th Australian Nitrogen Fixation Conference (ANFC) at the Esplanade Hotel in Fremantle.

The conference comes eight years after the very successful previous ANFC in Adelaide in 2014 and for many of us, particularly in Australia, it is the first in-person conference we have been able to attend for some time.

It has become something of a cliché to reflect on the rigours of the last few years and the impacts they have had on our lives, but with little doubt it has been an isolating time for many of us in science. This is particularly true in N_2 fixation research, where collaboration between microbiologists, plant scientists, agronomists, inoculant manufacturers and growers is essential to harnessing symbiotic N_2 fixation for the benefit of agriculture. This has been particularly challenging to manage with state and national border restrictions. So, it is great to be able to get together and exchange thoughts and ideas and mingle in a real, live, in-person conference again.

You will see from the conference program that the ANFC2022 will cover work across the whole gamut of symbiotic N_2 fixation research, from farming systems, nodule physiology, rhizobial metabolism, genomics, synthetic biology and inoculation practice. One of the strengths of the ANFC series is that the conference is "small enough" to avoid needing parallel sessions, thus allowing all of us to participate in oral and poster presentations that take us out of our intellectual comfort zone. Fresh eyes, mental frameshifts and drawing on the wealth of experience of an interested colleague can be a great catalyst for that "eureka" moment.

We are also heartened to see so many student presenters at this conference. Funding, laboratories, equipment, and field studies are all needed to tackle the constraints of symbiotic N_2 fixation and its application into the future, but we have no hope of tackling any of these challenges if we do not attract bright and inquiring minds to build a career in this field. The substantive representation from our student presenters bodes well for the future of N_2 fixation research.

Since the very first conference in 1955 in Sydney, the ANFC series has gained a reputation for providing a relaxed and informal atmosphere for colleagues to have open scientific debate. We therefore encourage you to continue to build on this tradition and contribute to the scientific program through discussions with your colleagues. N_2 fixation research is a collaborative and integrative field and one in which every step forward by any one of us, is a step forward for us all.

Jason Terpolilli, on behalf of the ANFC2022 Organising Committee

November 2022

ANFC2022 Organising Committee Members

Graham O'Hara, Helen Shortland-Jones, Yvette Hill, MacLean Kohlmeier, Ron Yates & Jason Terpolilli

Chronology of the Australian Nitrogen Fixation Conferences

Conference	Year	Location
1st	1955	Sydney, NSW
2nd	1963	Sydney, NSW
3rd	1966	Sydney, NSW
4th	1971	Canberra, ACT
5th	1975	Brisbane, QLD
6th	1979	Perth, WA
7th	1984	Sydney, NSW
8th	1986	Adelaide, SA
9th	1991	Canberra, ACT
10th	1993	Brisbane, QLD
11th	1996	Perth, WA
12th	1999	Wagga Wagga, NSW
13th	2002	Adelaide, SA
14th	2005	Katoomba, NSW
15th	2009	Margaret River, WA
16th	2012	Sydney, NSW
17th	2014	Adelaide, SA
18th	2022	Fremantle, WA

Acknowledgements

The Organising Committee would like to acknowledge the generous support from the Grains Research Development Corporation of Australia (GRDC), Department of Primary Industries and Regional Development (DPIRD) and Murdoch University's Food Futures Institute for their ANFC2022 sponsorship.

Conference Program

Mon 5th Dec		
15.00 – 18.00	Welcome session: conference sign-in at the Esplanade Hotel	
Tues 6th Dec	**Speaker**	**Title**
08.30 – 09.00	Welcome to Country by Richard Walley, Aboriginal Productions Conference Opening	
1. Legumes in Farming Systems I **Chair: Dr Giacomo Betti, Grains Research and Development Corporation (GRDC)**		
09.00 – 09.30	John Howieson Murdoch University	The "ascent of legumes" in farming systems: the critical roles of Wisdom, Weeds and Women
09.30 – 09.50	George Mwenda Department of Primary Industries and Regional Development WA (DPIRD)	The role of grain legumes in Western Australian farming systems
09.50 – 10.10	Tom Edwards Department of Primary Industries and Regional Development WA (DPIRD)	Increased efficacy of herbicides on Western Australia's sandplain soils can inhibit legume nodulation
10.10 – 10.30	Cameron Silburn Department of Agriculture and Fisheries, Queensland (DAF QLD)	At what soil nitrogen concentration is mungbean N_2 fixation inhibited?
10.30 – 11.00	**Morning Tea**	
2. Legumes in Farming Systems II **Chair: Ross Ballard, South Australian Research and Development Institute (PIRSA-SARDI)**		
11.00 – 11.30	David Herridge University of New England	N_2 fixation by legumes in pastures
11.30 – 11.50	Robert Harrison Department of Primary Industries and Regional Development WA (DPIRD)	Pasture legumes boost cereal proteins in dryland cropping systems
11.50 – 12.10	Liz Farquharson Department of Primary Industries and Regions - South Australian Research and Development Institute (PIRSA-SARDI)	Pulse Check: A critical assessment of pulse crop N_2 fixation in south-eastern Australia, highlighting limitations and opportunities
12.10 – 12.30	Ivan Kennedy University of Sydney	Export of agricultural produce a major source of increasing atmospheric CO_2: An important future role for biological nitrogen fixation
12.30 – 13.30	**Lunch**	

3. Legume Nodulation and Function I **Chair: Associate Professor Penny Smith, La Trobe University**		
13.30 – 14.00	Ulrike Mathesius Australian National University	Interactions of nodulation with nematode parasitism
14.00 – 14.20	Angus Rae Australian National University	New methods for confocal imaging of infection threads in crop and model legumes
14.20 – 14.40	Hayley Knights University of Oxford	Genome-wide identification of colonisation determinants in *Rhizobium leguminosarum* using random barcode transposon-site sequencing
14.40 – 15.00	Beatriz Jorrin University of Oxford	Stable, broad host-range fluorescent markers for tracking multiple bacteria on plant roots
15.00 – 15.30	**Afternoon Tea**	
4. Legume Nodulation and Function II **Chair: Professor David Day, Flinders University**		
15.30 – 16.00	Brett Ferguson University of Queensland	Molecular mechanisms of legume nodulation control
16.00 – 16.20	Pinhui Wang Australian National University	The identification of flavonoids that modulate polar auxin transport during root nodule development in *Medicago truncatula*
16.20 – 16.40	Jessie Dolliver University of Oxford	Mechanisms of bacterial primary attachment to plant roots under differing pH conditions
16.40 – 17.00	Clare Cocker University of Oxford	A master regulator of primary attachment to pea roots
17.00 – 20.00	**SUNDOWNER AND POSTER SESSION I** King Sound and Prince Regent Rooms, Esplanade Hotel	

Wed 7th Dec	Speaker	Title
5. Physiology and Genetics of Symbiosis I **Chair: Dr Jason Terpolilli, Murdoch University**		
08.30 – 09.00	Philip Poole University of Oxford	Adaptation, selection and sanctioning in the Rhizobium-legume symbioses
09.00 – 09.30	Penelope Smith La Trobe University	Iron transport in nodules – some key players identified but questions remain
09.30 – 09.50	Georgina Stagg Murdoch University	Genetic determinants of host range in *Mesorhizobium ciceri* strains WSM1271, WSM1497 and WSM1284
09.50 – 10.10	Raphael Ledermann University of Oxford	Polyamines are essential for bacteroid maintenance and N_2-fixation
10.10 – 10.30	David Day Flinders University	Nodulin-26 is a multifunctional plant aquaporin that facilitates ammonium transport in nitrogen-fixing soybean nodules
10.30 – 11.00	**Morning Tea**	
6. Physiology and Genetics of Symbiosis II **Chair: Dr Josh Ramsay, Curtin University**		
11.00 – 11.30	Ivan Oresnik University of Manitoba	Relationship between central metabolism and nitrogen fixation in *Sinorhizobium meliloti*
11.30 – 11.50	Talitha Rogers Murdoch University	The physiology, metabolism and energetics of respiratory chains in *Paraburkholderia sprentiae* WSM5005, in free-living and symbiotic conditions
11.50 – 12.10	Thomas Underwood University of Oxford	Modelling the legume-Rhizobium symbiosis
12.10 – 12.30	Rebecca Fudge University of Minnesota	Tracking N_2 fixation efficiency effects on plant and nodule growth
12.30 – 13.30	**Lunch**	
7. Evolution and Diversity of Rhizobia I **Chair: Dr Liz Farquharson, South Australian Research and Development Institute (PIRSA-SARDI)**		
13.30 – 14.00	Macarena Gerding-Gonzalez Universidad de Concepción	The role of root nodule bacteria in the recovery of *Sophora toromiro*: The extinct Easter Island legume tree
14.00 – 14.20	Yvette Hill Murdoch University	Novel root nodule symbionts of *Cicer arietinum* grown in the Ord River Scheme

14.20 – 14.40	Judith Rathjen University of Adelaide	Diversity and field symbiotic performance of *Mesorhizobium* strains collected across chickpea cropping areas of Australia and Myanmar
14.40 – 15.00	MacLean Kohlmeier Murdoch University	Genomics of Australian commercial inoculants
15.00 – 16.00	**Afternoon Tea and POSTER SESSION II**	
16.00 –	**Free afternoon/evening**	

Thurs 8th Dec	Speaker	Title
8. Evolution and Diversity of Rhizobia II **Chair: Professor Philip Poole, University of Oxford**		
08.30 – 09.00	Joshua Ramsay Curtin University	Diverse populations of nonsymbiotic *Mesorhizobium* spp. present in soils have a capacity to become legume symbionts following horizontal gene transfer
09.00 – 09.30	Tim Haskett University of Oxford	Engineering nitrogen fixing symbiosis between cereals and bacteria
09.30 – 09.50	Craig Wood Commonwealth Scientific and Industrial Research Organisation (CSIRO)	Progress towards engineering nitrogenase directly into crops
09.50 – 10.10	Callum Verdonk University of Western Australia	A superhelical-filament-forming DNA-binding protein controls horizontal transfer of symbiosis genes
10.10 – 10.30	Tahlia Bastholm Curtin University	Bacterial cell-cell signalling regulates a network of non-coding RNAs in *Mesorhizobium japonicum* and *M. ciceri*
10.30 – 11.00	**Morning Tea**	
9. Inoculation Technologies and Practice I **Chair: Dr David Herridge, University of New England**		
11.00 – 11.30	Jason Terpolilli Murdoch University	Securing rhizobia germplasm of global value
11.30 – 12.00	Ahmed Hassen Agricultural Research Council – Plant Health and Protection (ARC-PHP)	Persistence of soybean *Bradyrhizobium* inoculants in the soil and impacts on soil microbial diversity as determined by soil nodulation trapping and shotgun metagenomics studies
12.00 – 12.20	Kit Burns Murdoch University	Cross-compatibility and genetic stability of rhizobia to maximise nitrogen fixation in the new annual pasture legume *Scorpiurus muricatus*
12.20 – 13.30	**Lunch**	

10. Inoculation technologies and practice II **Chair: Professor John Howieson, Murdoch University**		
13.30 – 14.00	Ron Yates Department of Primary Industries and Regional Development WA (DPIRD)	Replacement of WSM1455 (Group E/F) with WSM4643 for *Pisum sativum*, *Lens culinaris* & vetch (*Vicia sativa* and *V. villosa*)
14.00 – 14.30	Jessica Rigg/Graham O'Hara Australian Inoculants Research Group/ Murdoch University	Assuring high quality of legume inoculants: maximising the potential of legume inoculants for N_2 fixation from the start
14.30 – 15.00	Ross Ballard Department of Primary Industries and Regions - South Australian Research and Development Institute (PIRSA-SARDI)	Use of DNA tests to quantify the number of *Rhizobium leguminosarum* bv. *viciae* and *Mesorhizobium ciceri* in field soils of varying pH
15.00 – 15.30	**Afternoon Tea**	
15.30 – 16.30	Prizes Closing Remarks	
18.30 – late	**CONFERENCE DINNER** Bathers Beach House, Fremantle	

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SESSION 1 – LEGUMES IN FARMING SYSTEMS I

Chair: Dr Giacomo Betti, Grains Research and Development Corporation (GRDC)

The "ascent of legumes" in farming systems: the critical roles of Wisdom, Weeds and Women.

John Howieson
Legume Rhizobium Sciences, Murdoch University, Murdoch, Western Australia

Key words
Legumes, adoption, wisdom, weeds, women

Despite the well demonstrated advantages that legumes confer upon mixed farming operations, their adoption adds layers of complexity to management, and is thus not axiomatic. In the Western Australian context, breeders successfully domesticated then altered the chemical constituents of *Lupinus angustifolius* to meet the food market. A sustained effort was made to breed a unique combination of protein, lipids and fibre and then to market them as a "health food" to increase their net value to growers. For the pasture legumes, adoption barriers were identified relating to the price of seed, perceived poor feed availability during the establishment year, and labour conflicts during autumn. Summer sowing of header harvested seeds was invented following an acute understanding of hard seed breakdown behaviour. Despite these substantial advances, adoption levels of the legumes remain low. This essay unravels the critical roles of the three Ws: "Wisdom, Weeds and Women" in affecting the adoption rates of legumes into modern farming systems.

The critical role of Wisdom: Legumes are actually niche species, and they require their optimal niche to maximise growth, N_2 fixation and reproduction. Without optimal performance, legumes are often inferior to other choices of plants to cultivate and are therefore not selected by farmers. Consistent with this, it is important to acknowledge that not all legumes are, in fact, adapted to infertile soils. The best example to illustrate this is in the attempts between 1970 and 2020 to develop a wider range of pulse legumes for the mildly acid sands and sandy loams of WA. None of *Cicer, Vicia, Lens* or *Pisum* species grow well enough on most of the WA soils to merit inclusion in a farming system. The infertility factors in this scenario – mild to moderate acidity and low CEC, low C and poor water holding capacity – conspire to defeat attempts to grow these pulses profitably, even though they are favoured elsewhere globally. Even the most well adapted grain legume for WA, lupin, is only well adapted to approximately 1 million of the 15 million ha of farmland in the SW of WA, and it is only now grown on those specific deep sands in which its niche is found. *Ornithopus* is favoured on the less fertile acid sands, with infertility in this context usually defined by clay content below 3%, organic C below 1% and a soil depth of less than 0.5 m.

The accumulation of *Wisdom* about the optimal niche for each legume is essential if it is to be considered for adoption. In the WA context again, specific niches are now recognised for lupins, the pulse genera mentioned above, and the pasture legumes *Ornithopus, Biserrula, Trifolium*

subterraneum, T. balansa, T. spumosum, T. glanduliferum, Trigonella, and four spp. of *Medicago*. The niches barely overlap, and to identify the right legume for the right niche requires a substantial accumulation of *Wisdom* by farmers, scientists and consultants.

The critical role of Weeds: In WA, after a general appreciation of the niche requirements for legumes, optimal genetics were acquired for many of those listed above. It was shown that farmers could grow a high yielding, high protein cereal crop without providing industrial N after a single year of *Ornithopus* cultivation. Similarly, the systems benefits of lupins were clarified, their diseases managed and robust agronomic packages developed. With Urea prices rising, one would have expected automatic adoption of such well adapted grain and pasture legumes. This hasn't eventuated, with lupin sowings falling from an historic high of 2 million ha pa to several hundred thousand in 2022, and great regions of wheatbelt WA with poor, unimproved pastures. The reasons for this are complex, but a main factor is an inability to control *Weeds* in the legume phase. Farmers have spent the last 50 years removing every *Weed* from their crops, following adoption of minimum tillage, with assiduous application and rotation of herbicides. This aversion to *Weeds* is not because they compete with crops for moisture and nutrients, but more so that the presence of some *Weeds* lowers the market value of the crop (dramatically more than their competition affects the yield of the crop). *Weeds* are easy to remove from cereal and canola crops as the international chemistry has been focussed upon this. But they are more difficult to remove from legumes. Nor are they even necessary to remove from legume pastures as their presence often improves the nutritive value of pastures to grazing animals. So, there is a direct conflict in the value of pastures *per se* and the wish to have the following crops completely *Weed* free. *Weed* management is a major constraint upon legume adoption, despite the fact that the top 10% of farmers can achieve it if they wish to.

The critical role of Women: Until very recently in the ascent of man "it was the role of women to produce he-children and to prepare the meals" (Bronowski 1970). However, in modern Agriculture, it is the *Women* who are often the innovators (Alston 2010). Why do we raise the role of *Women* when discussing the adoption of legumes? In sub-Saharan Africa, the *Women* predominantly tend the crops and still prepare the meals. It was the *Women* we worked with in N2Africa that first saw the benefits of soybean to soil fertility, and indeed to the nutrition of their infants. It was therefore the *Women* who were able to convince the men of the benefits of soy, despite it being outside their normal diet (and hence appreciation as a food). In WA, lupins have been shown to be the very best of health foods, but again it is overwhelmingly *Women* who are the innovators in access to healthy diets. If we are to bring new foods into the marketplace, then it is the *Women* who will have the first say in their adoption. For the pasture legumes, in contrast to the experience in Africa, in mixed farming enterprises in WA it is often the role of *Women* to manage the stock (while the men manage the cropping program). Further, the *Women* are often managing the books and the marketing and accessing *Wisdom* through the information platforms with respect to optimising farming systems. Although in our experience it can take a decade of quiet persistence, it is often the *Women* who need to firstly convince the men to improve the pastures in WA with legumes, despite the obvious benefits to stock, to rotations and to the bottom line.

Conclusions: Barriers to adoption of legumes in farming systems are complex and they are not easily overcome. Despite successful efforts to develop well adapted legumes for nearly every soil niche in WA, large swathes of the wheatbelt are farmed without them. Despite the economic and biological advantageous of incorporating legumes into these farming systems, it is only where acute knowledge of net benefits and best practices co-exist that we see adoption barriers removed. Understanding the role of the three Ws will accelerate this.

References

Bronowski, J (1973) The Ascent of Man. Little, Brown and Co., Boston, USA.

Alston, M (2003) Women in Agriculture: the 'New Entrepreneurs'. *Australian Feminist Studies* **18**(41): 163-171.

The role of grain legumes in Western Australian farming systems

George Mwenda[1] and Steve Davies[2]

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Key words

Pulses, nitrogen fixation, soil-borne diseases, break crops, soil amelioration

Abstract

Pulses play a critical role in farming systems. They provide nitrogen to soils via their unique ability to fix nitrogen, reduce cereal pests and diseases, and can mitigate against financial risks through diversification. However, despite these widely known benefits, the adoption of pulses in Western Australia remains very poor. Barriers to adoption include inadequate access to infrastructure and markets, insufficient grower knowledge and technical skills, concerns around reliability and profitability, and poor adaptation of legumes and rhizobia to Western Australian soils.

Recent advances in pulse genetics, acid-tolerant rhizobia, weed control options, and soil amelioration can potentially increase the adoption rate of pulses in WA. For example, pulse options on deep sands in Western Australia are often limited by the poor adaptation of many legume species and rhizobia to low soil pH, clay content, or both, but with the ongoing widespread adoption of soil amelioration in WA, there is an expectation that amelioration, including the incorporation of lime to increase soil pH, will make them suitable for a broader range of pulse species, such as chickpeas, faba beans and lentils. Growers could also benefit from enhanced technical and extension programs, policy support, and infrastructure and market development.

Increased efficacy of herbicides on Western Australia's sandplain soils can inhibit legume nodulation

Tom Edwards[1,2], Ronald Yates[1,2], Stephen Davies[1], Mick Rose[3], John Howieson[2], Graham O'Hara[2], Emma Steel[2], Chris Poole[2], Robert Harrison[1,2], Benedict Arthur[2] and David Hall[1]

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Key words

Herbicides, sandy textured soils, nodulation, legumes

Summary

We will present data from five field trials and six glasshouse studies that illustrate:

- Biological N_2 fixation from legumes is underperforming on sandplains soils of Western Australia
- Biological availability and persistence of several commonly used herbicides are increased on sandy acid soils
- The process of legume nodulation by rhizobia is identified as being particularly sensitive to herbicide residues on acid sandy soils

Introduction

Biologically fixed nitrogen (BNF) through the legume-rhizobia symbiosis is an integral component of Australian farming systems. Howieson and Herridge [1] estimated that 80% of the nitrogen in Australian grain was produced by legumes which contributed AU$3 billion saving on synthetic nitrogenous fertilisers. Critically, on Western Australian sandy textured soils BNF achieved is often well below optimal levels and an unreliability of nitrogen benefit in subsequent crops is seen as a barrier to further adoption.

Western Australian sand plain soils are dominated by acid infertile sands with <5% clay and <1.5% organic matter in the topsoil, and these soils cover more than 10 million hectares [2]. The fragility of sandy textured soils has precluded mechanical methods of weed control. As a consequence, there is a complete reliance on the increased use of herbicides to enable optimal crop production [3]. To better understand how increased herbicide use may interact with BNF on sand textured soils, we have initiated five field trials and six glasshouse studies.

Discussion and Results

The physical composition of the soil (percentage of sand, silt clay and organic matter) and the chemical properties of the soil (pH, nutrients, cation exchange capacity) strongly influence the soil adsorption of herbicide [4]. These properties combined with soil biology and climatic conditions also influence herbicide uptake and degradation. Modest levels of organic matter and clay particles equate to the scant attenuation of herbicides on sandy textured soils. Therefore, potentially a higher concentration of herbicide is biologically available after application on these soils (Table 1). Additionally, these soil

properties, combined with low rainfall in a Mediterranean climate, confer a relatively slow rate of herbicide degradation through both microbial and chemical pathways.

The legume-rhizobia symbiosis is particularly sensitive to many commonly applied herbicides and is impeded at concentrations that do not impact other components of plant production (Table 1). Therefore, we believe that an increased herbicide persistence and biological availability of commonly applied herbicides on sandy textured soils is significantly reducing BNF in circumstances where there is no measurable reduction in plant performance (Table 2). This indicates that research is required to further illuminate the specific mechanisms that are disrupted in the legume-rhizobia symbiosis and to develop strategies for herbicide use that reduce the impact on biological nitrogen fixation on these sandy textured soils.

Table 1. Effective dose of soil applied herbicide Trifluralin (ai g/ha) for a 50% reduction in nodule score and top dry weight of *Lens culinaris* cv Bolt. Each soil was sampled in-situ from a range of field sites across the Western Australian wheatbelt.

SITE	LOAMY CLAY K	LOAMY CLAY G	GER	ESP	EMB	GMB
CLAY %	10	10	3	2	2	4
ORGANIC CARBON %	1.3	2.1	0.4	0.9	0.6	0.3
NODULATION ED50 AI G/HA	494	2600	621	390	91	260
TOP DRY WEIGHT ED50 AI G/HA	595	3000	994	340	115	637

Table 2. Percentage of nitrogen derived from atmosphere resulting from a combined treatment sample on nine legume cultivars grown in soil with different herbicides (Chlorsulfuron, Triasulfuron, Clopyralid, Pyroxasulfone and control: no herbicide) applied to wheat 14 months prior to data collection in Brookton, WA. (SS)= summer sown

	Legume cultivar								
Herbicide Treatment	Bartolo	Bartolo (SS)	Margurita	Margurita (SS)	Prima	Dalkeith	Casbah	Mandelup	Kaspa
Clopyralid (115g/ha)	40.3	34.5	45.0	34.2	72.7	24.5	47.7	86.1	81.2
Chlorsulfuron (15g/ha)	9.5	7.4	62.8	21.7	61.4	49.3	75.4	90.3	75.7
Triasulfuron (35g/ha)	44.5	10.1	31.9	23.9	70.7	57.4	53.2	76.0	80.1
Pyroxasulfone (125g/ha)	40.4	15.1	41.9	28.6	49.0	31.4	65.2	79.9	78.5
Control (no herbicide)	61.9	47.2	59.7	39.6	71.5	57.5	77.5	89.9	80.2

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At what soil nitrogen concentration is mungbean fixation inhibited?

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Queensland Department of Agriculture and Fisheries, Brisbane City, Queensland

Key words
Mungbean, nitrogen, rhizobia, %Ndfa

Introduction

Mungbean is the main summer pulse crop grown in Australia with 95% of the total production exported to meet overseas demands for the grain. Total mungbean production has increased from 20 000 t/ha in 1996 to 90 000 t/ha in 2020. One benefit of planting mungbeans is their ability to fix nitrogen from the atmosphere to meet nitrogen demands. Currently, most growers inoculate mungbean crops with commercial rhizobia to promote nodulation and nitrogen fixation, which is believed to supply adequate N for that crop. However, past research has indicated that inoculated mungbeans were nitrogen-limited because of the suppressive effects of nitrate and/or insufficient numbers of rhizobia (Herridge et. al. 2005). Research for all legumes shows that the proportion of N in the plant derived from fixation decreases with increasing levels of soil mineral N. Levels that impact N fixation in chickpea and soybean have been estimated from experimental work (Doughton et al. 1993; Salvagiotti, 2008). Anecdotal evidence suggests that the threshold level of mineral N is lower for mungbean than for other legumes, however, the point at which nodulation starts to be reduced and then inhibited for this species has not been established. A glasshouse pot experiment under controlled conditions was conducted in 2021 to determine the level of soil nitrogen where mungbean nodulation and fixation is reduced and totally inhibited, and how this affected biomass. It was hypothesised that: (a) nodulation and fixation would be at their highest levels when soil mineral N is at its lowest and declines as soil nitrogen increases; (b) nodulation and fixation will be totally inhibited at a particular soil mineral nitrogen concentration; and (c) biomass will be influenced by the relationships of soil nitrogen, inoculation, and fixation.

Results

Soil N concentration levels at planting influenced both nodule number and dry weight of inoculated mungbeans. Lower soil N concentrations at planting resulted in the highest nodule numbers which also positively correlated with nodule dry weight. As N concentrations increased, nodule number and weight decreased. At the highest N concentrations there were still nodules found on the mungbean roots, albeit in very low numbers. As applied soil N increased the amount of N fixed (%Nitrogen derived from atmosphere; %Ndfa) decreased (Figure 1). Inoculated mungbeans fixed between 95 and 87 %Ndfa when N concentrations ranged from 0 to 20 kg N/ha, then decreased rapidly until 80 kg N/ha was applied , stabilising at around 42 to 35%Ndfa at concentrations 150 kg N/ha (Figure 2). Amount of N fixed decreased by over a half to 19 kg N/ha when soil N rates were above 80 kg N/ha. Inoculated and uninoculated mungbean biomass responded differently to increasing levels of applied N. Inoculated mungbeans showed very little change in above ground biomass across all applied N rates. In comparison, the uninoculated showed a significant increase in above ground biomass up to 60 kg

N/ha. After which biomass remained relatively stable. Root biomass followed a similar trend to above ground biomass.

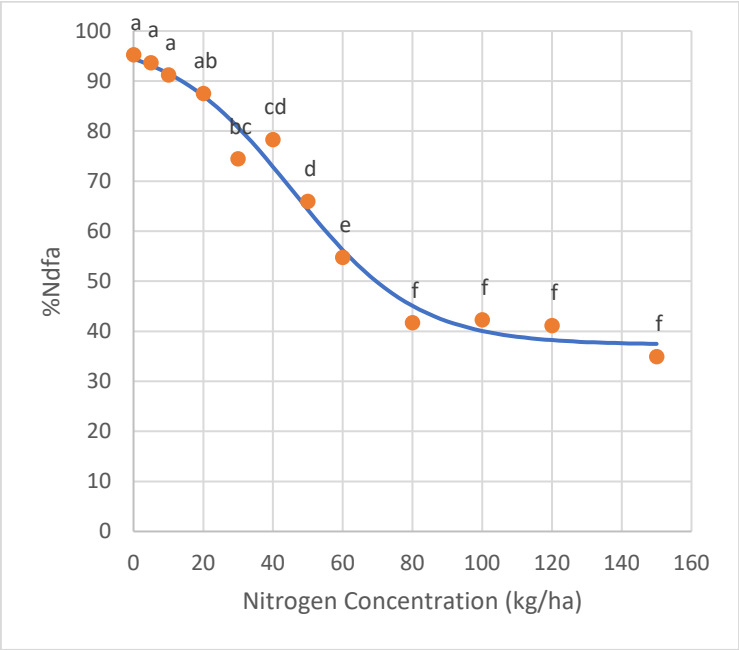

Figure 1. Percentage nitrogen derived from the atmosphere (%Ndfa) 34 days after planting mungbeans (cv. Jade-AU) that were inoculated with rhizobia (CB01015). Fitted with a logistics curve using average values.

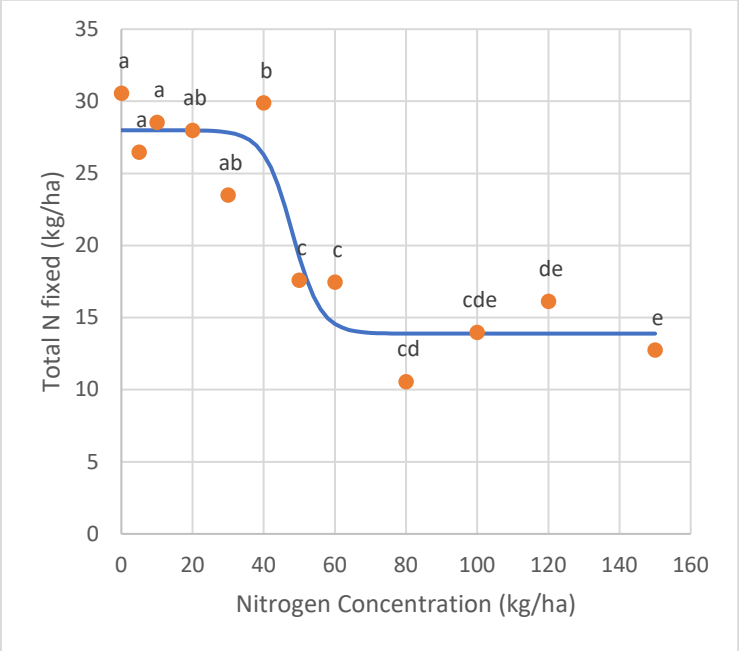

Figure 2. Total N fixed (kg/ha) per pot 34 days after planting mungbeans (cv. Jade-AU) that were inoculated with rhizobia (CB1015). Fitted with a logistics curve using average values.

Discussion

Mungbean nodulation and fixation was significantly negatively correlated with soil N concentrations as hypothesised; as soil N increased, nodulation activity decreased. Pampana, Masoni et al. (2018) and Doughton, Vallis et al. (1993) also report similar trends with several other legume crops. They also found a negative relationship between soil N concentrations and nodulation as well as N fixation in legume crops. The point at which mungbean nodulation and fixation starts to become inhibited is when soil N concentrations reach 40 kg N/ha, however fixation and nodulation is not totally inhibited at the highest concentrations we used. This disproves the hypothesis that mungbeans will stop fixing atmospheric N at the highest N concentrations in this trial. This is also supported by several other studies for different legumes which showed low fixation when grown in very high soil N concentrations (Doughton et al. 1993; Turpin, Herridge et al. 2002). This research has shown where mungbeans source their N from up until the vegetative stage; either the mineral N pool or fixation and how this varies across ranging soil N concentration. It has shown that mungbeans will mostly rely on fixation up until 40 kg N/ha in the soil, after which will rely on the soil mineral N pool more. This could have serious farming system impacts when deciding on N budgets for the mungbean crop as well as future crops in the rotation. Some growers and agronomists could be over- or underestimating how much mungbeans are using soil N in their system.

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SESSION 2 – LEGUMES IN FARMING SYSTEMS II

Chair: Ross Ballard, Department of Primary Industries and Regions South Australia (PIRSA) – South Australian Research and Development Institute (SARDI)

Nitrogen fixation by legumes in pastures: the major nitrogen input to underpin Australian farming

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Key Words
Legume nitrogen fixation

Introduction

Knowledge of the rates and total amounts of nitrogen (N) fixed by leguminous pastures and crops is necessary to fully contextualise the critical role that legumes play in Australian agriculture. For the individual farmer, that same knowledge at the paddock level creates opportunities for he or she to manage the supply of N for grain and livestock production more effectively. During the past 50 years, enormous progress has been made to develop methods for quantifying N_2 fixation by field-grown legumes, much of which involved Australian scientists. In concert with this, Australian scientists were also active in compiling and aggregating the thousands of plot and paddock estimates to generate values and functions that could be used to quantify N_2 fixation at broader scales (e.g. Peoples et al. 2012; Unkovich et al. 2010; Angus and Peoples 2012).

Published N_2 fixation data and area and production statistics relevant to the Australian livestock and grains industries (e.g. ABS 2019) were combined to determine 130-year timelines of pasture- and pulse-legume fixed N inputs and more detailed situation profiles.

Methodology

Accurate accounting of the N fixed by grazed pastures in Australia was, and remains, extremely challenging, the main reason being uncertainty about the productivity of the 336 Mha of pastures and the fraction of those pastures that is legume. Nonetheless, estimates were made by combining pasture areas, types and legume fractions across 72 Mha (Donald 2012) with annual legume and lucerne water-use efficiencies (WUE) (21 references) with rainfall for 2012–14 (M Unkovich, pers. comm.) to estimate average, yearly total pasture and pasture legume shoot DM production. Outputs were then merged with ABS statistics for all pastures (336 Mha) and N_2 fixation rates at state and national scales estimated using coefficients for %N shoot, below-ground N factor and %Ndfa (Unkovich et al. 2010; Peoples et al. 2012). For the pulses, ABS/ABARES statistical data at state and national scales were combined with established coefficients for harvest index (HI), %N shoot, below-ground N factor and %Ndfa to estimate N_2 fixation rates (Peoples et al. 2021; Herridge et al. 2022).

Timeline of legume fixed N inputs

Pastures were divided into subgroups – improved legume-based (producing 2.0 t/ha legume shoot DM/yr and fixing 85 kg N/ha/yr), improved grass-based (0.5 t/ha legume shoot DM/yr and fixing 20 kg N/ha/yr) and rangelands (0.1 t/ha legume shoot DM/yr and fixing 4 kg N/ha/yr). The pulses were estimated to fix N at a rate of 90 kg/t grain. A timeline of N_2 fixation for the 130 years (1890–2020) was then constructed using those rates, with pastures adjusted each year for rainfall, and combined with area statistics from the ABS and other sources (Figure 1).

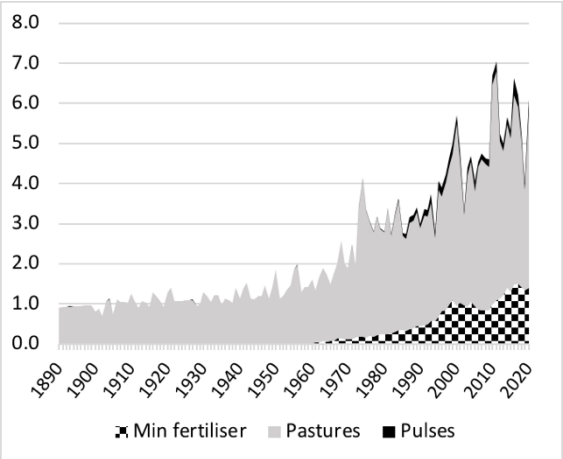

Figure 1. Timelines of estimated annual inputs of mineral fertiliser N and legume fixed N by pastures and pulses (million t/yr) in Australian farming during 1890–2020.

Between 1890 and 2020, an estimated 270 million t N was fixed by pasture and pulse legumes in Australia's farming systems (Table 1). During the same period, about 37 million t mineral fertiliser N was used in Australia. Currently, an estimated 80% of N inputs are by nodulated legumes vs 20% from fertiliser.

Table 1. Estimated annual, average and cumulative inputs of N fixed by the improved pastures, rangelands and pulses in Australia during 1890–2020. Assumed rates of N_2 fixation were 60 kg N/ha/yr for improved legume+grass-based pastures, 4 kg N/ha/yr for rangelands (adjusted for rainfall) and 90 kg N/t grain for pulses.

Timeframe	Area (million ha)			N_2 fixation (million t)			
	Improved	Rangelands	Pulses	Improved	Rangelands	Pulses	Grand total
Annual							
1890	4.6	158	0.0	0.28	0.63	0.00	0.9
2020	50	290	2.0	3.20	1.23	0.27	4.7
Average							
1890–2020	17	240	0.5	1.03	0.96	0.06	2.1
Cumulative							
1890–2020				134	127	7.2	268

Conclusion

The legion of dedicated Australian (and other) scientists, farmers and extension personnel who observed, researched, and promoted nodulated legumes during the past 130 years to make them so extraordinarily beneficial for Australia's farming systems are acknowledged.

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Pasture legumes boost cereal proteins in dryland cropping systems

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Key words

Crop rotation, nitrogen fixation, synthetic N reduction

Abstract

Reducing synthetic inputs in farming systems is paramount for the sustainable success of the agri-food industry. A major input in dryland farming systems is nitrogen (N) fertiliser, which until recently was relatively inexpensive. This has resulted in an over-reliance on fertiliser N in southern Australia, which has caused a depletion of soil N reserves. Furthermore, the recent cost of fertiliser N globally has increased both financially (i.e. reduced on-farm profit) and environmentally.

The southern cropping region produces 3.5% of the world's wheat (AEGIC, 2022), however, the quality (% protein) has been diminishing as a consequence of the lack of organic N fixed by pasture legumes in rotation in farming systems (Lemon 2007). Additionally, it was perceived that the traditional pasture legumes (e.g. *Trifolium subterraneum*, *Medicago* spp.) were not climatically adapted to this changing region. Therefore, in the last 20 years, new pasture legumes (e.g. *Trigonella balansae, Ornithopus* spp.) have been bred with the ability to fix more N_2, have deeper root systems and have a higher tolerance to drought conditions than the traditional species (Loi et al. 2005; Nutt et al. 2021). Although, there is minimal knowledge of how these next generation pasture legumes perform in a modern farming system. Therefore, the aim of this research was to determine if biologically fixed N_2 from elite pasture legumes can increase grain protein levels in dryland farming systems across multiple soil types in WA.

Extensive field trials containing fallow, continuous cereal and legumes such as *Ornithopus sativus*, *Vicia sativa*, *M. polymorpha* and *T. balansae* were studied in rotation with cereals at three markedly different soil types and climates (across dryland Western Australia) between 2018-2021. All ex-legume treatments in rotation sustained or exceeded cereal protein levels of continuous cereal rotations with less synthetic N (units/ha) applied. Although, cereal protein levels were diluted and lower in 2021 the ex-legume plots were still significantly higher than continuous cereal plots. Also, efficient N_2-fixing pasture legumes used in rotation with cereals can allow farmers to hedge on undersupplying N without significantly compromising yield and protein in high demand years, while reducing the chance of oversupply in years of low demand.

Prices of N have increased 175% over the 5-year average (Ostendorf 2021) and it is estimated that synthetic N application can contribute as much as 50% of the CO_2 emissions from a farm system (Camargo et al. 2013). If farmers got paid a higher premium for high grain protein content, this could encourage greater use of well-managed pastures, improved soil health, and higher sustainability of the farming system.

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Pulse Check: A critical assessment of pulse crop N_2 fixation in south-eastern Australia, highlighting limitations and opportunities.

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Key Words

Nitrogen fixation, nodulation, faba bean, field pea, chickpea, lentil

Introduction

The area of pulses grown in Australia continues to expand due in part to (i) the increased global demand for plant based proteins, which is expected to be worth $US16.9 billion by 2026 (Mordor_Intelligence 2022); and (ii) the ever-increasing cost of fertiliser N, with global urea prices increasing by 200% in the past two years. For these reasons, maximising N_2 fixation by pulses in farming systems to boost both grain protein production and soil N benefits is critical to achieving not only farm productivity and profitability but also supporting farming system sustainability.

In a 10-year period between 2011 and 2021, N_2 fixation has been measured in 50 replicated field trials, conducted primarily in South Australia and Victoria. Crops were sampled at mid-pod fill to determine maximum crop dry matter (DM) production, and N_2 fixation using the ^{15}N natural abundance method (Unkovich et al. 2008). Detailed meta data including plant genotype and treatment details, nodule number per plant, crop biomass (at mid-pod fill) and yield has also been collected. We compared the average and optimum (upper boundary) relationships between legume shoot biomass and N_2 fixation, based on the concept of water-use efficiency (French and Schultz 1984), to estimate potential rates of N_2 fixation for each of the four pulses – faba bean (bean), lentil, field pea (pea) and chickpea – and the degree to which the pulses are currently performing.

Results and Discussion

Average values for shoot N fixed at mid-pod fill were 94 kg/ha for faba bean, 83 kg/ha for pea, 54 kg/ha for lentil and 39 kg/ha for chickpea with values for shoot N fixed and shoot DM highly correlated (r^2>0.9) for all species (Figure 1). Given the amount of N fixed is strongly influenced by shoot DM production (Peoples et al. 2009) this is not a surprising result. That said, at a given level of DM, the amount N fixed within each species varied substantially, and particularly so for field pea. Comparison of the species regression lines with the upper boundary (potential) of each crop showed the average efficiency for each crop ranged from 82% for bean to about 70% for pea, lentil and chickpea (Table 1). The slope of the regression line was higher for bean than for lentil, chickpea and pea, partly due to the increased frequency of below average values in lentil and pea indicating greater potential to improve these symbioses.

Table 1. The slopes and regression coefficients (r^2) of lines of best fit describing relationships between shoot dry matter (t/ha) and shoot N fixed (kg/ha) for the 4 pulses. The performance of each symbiosis (% of optimum) was calculated using the average and upper boundary values for shoot N fixed, for shoot DMs of 5.0 t/ha. Percent optimum was calculated as (average shoot N fixed/upper boundary shoot N_2 fixed)*100.

Species	Slope	r^2	N_2 Fixed (% Optimum)
Bean	20.02	0.96	82
Chickpea	16.80	0.92	71
Lentil	17.40	0.91	70
Pea	16.39	0.92	68

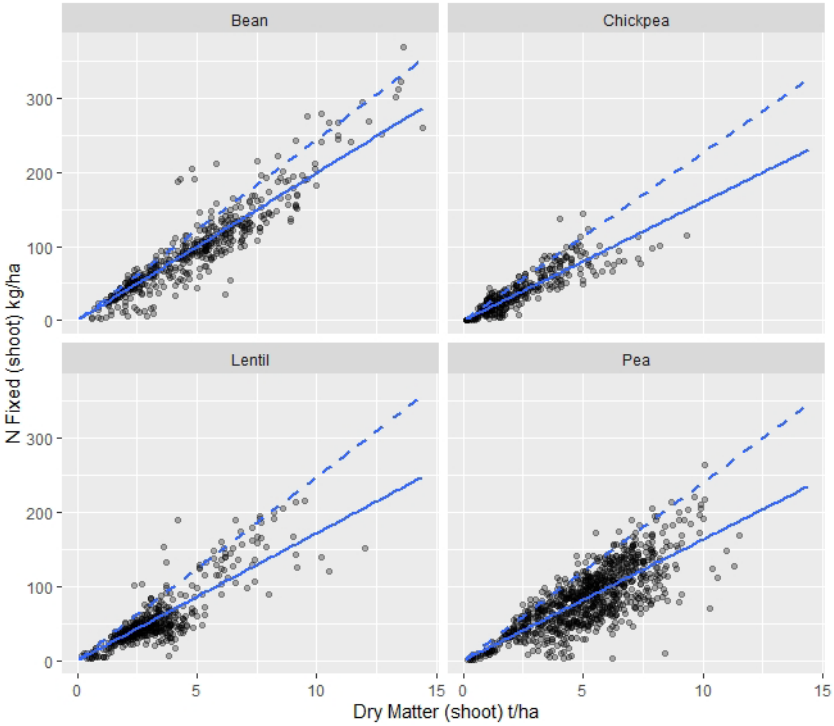

Figure 1. Relationships between shoot dry matter (t/ha) and shoot N fixed (kg/ha) for faba bean (n=465), lentil (n=499), field pea (n=933) and chickpea (n=351) in replicated field trials across South Australia and Victoria between 2011 and 2021. Each dot represents an individual measure at the replicate plot level within a replicated field trial. The solid line is the line of best fit of the data, determined by regression analysis; the dashed line represents the upper boundary of the data.

In this paper we will tease apart some of the underlying factors that may be responsible for sub-optimal N_2 fixation such as hostile (particularly acidic) soils, high populations of ineffective but infective soil rhizobia outcompeting the inoculant rhizobia or elevated soil nitrate levels suppressing nodulation and N_2 fixation.

Acknowledgements

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Export of agricultural produce a major source of increasing atmospheric CO_2: An important future role for biological nitrogen fixation

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Keywords

Keeling curve, soil acidification, CO_2 emissions

Abstract

The export of agricultural produce from rural to urban environments represents an increasing source of loss of nutrients and alkalinity from soils as the global human population increases. The application of increasing amounts of nitrogenous fertiliser also acidifies soils and the atmosphere (Kennedy, 1992). Even alkaline soils well buffered with bicarbonate ions up to pH 8 will contribute CO_2 in almost stoichiometric amounts when strong acid is added to the soil solution. Surprisingly, this source of CO_2 emission is not considered in the IPPC's global climate models, even though as a biological source like fossil fuels it is also depleted in ^{13}C-content.

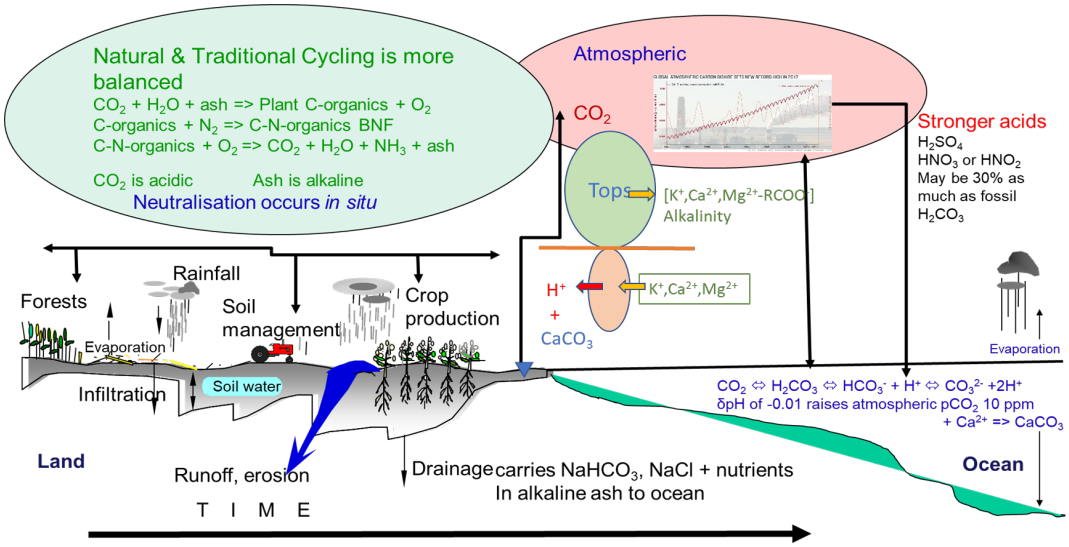
The models for CO_2 in the atmosphere shown in the Keeling curve measured on Mauna Loa consider the increase as caused by kinetic imbalances between C-assimilation by photosynthesis, and emission by combustion of fossil fuels and biosphere respiration. Even though the alkaline oceans are estimated to absorb perhaps half fossil fuel emissions, it is speculated that lack of soil nutrients may mean ecosystems lack capability to absorb the remainder biologically. However, little account is taken of physico-chemical factors such as temperature and pH values, since equilibrium is discounted. Soils below pH 5 contain very little bicarbonate in solution, but addition of limestone by farmers to raise soil pH to increase fertility can add an additional amount of CO_2 to overcome acidity, though wrongly rejected in the literature as a source of significant emissions.

Table 1. Equivalents of acid production in global ecosystems, with estimates per square metre.

Acid	Source	Moles/year	H^+ meq/m^2
Strong acids			
H^+	Croplands $42x10^8$ ha	$28x10^{12}$	54.9
H^+	Forestry $40x10^8$ ha current	$16x10^{12}$	31.4
H^+	Pasturelands $30x10^8$ ha	$3x10^{12}$	5.9
HNO_3	Nitrification of NH_3 legume-N	$14x10^{12}$	27.5
H_2SO_4	Coal, oil, gas, wood	$10x10^{12}$	39.2
H_2SO_4, H_2SO_3	Anaerobic sulfate respiration?	$25x10^{12}$	98.0
HNO_3, H_2SO_4	Wildfires	$12x10^{12}$	25.9
			282.8
Weak acids			
H_2CO_3	Global respiration, weak acid	$17,500x10^{12}$	34,307.0
H_2CO_3	Coal, oil and gas emissions	$816x10^{12}$	1,599.7
H_2CO_3	Wildfires	$100x10^{12}$	392.1
H_2CO_3	Cement, construction, weak acid	$53x10^{12}$	104.6
Total H^+ emissions	Estimate only ±10%		36,261.3
Weak acid	H_2CO_3 (pK 6.5)		35,947.2

The exact scale of these processes acidifying soil and releasing CO_2 is unclear. Some rough estimates based on data from UN and FAO sources are indicated in Table 1 for the processes and also indicated in the figure below. The content of carbon in soils has been estimated as several times that of the atmosphere's CO_2 and it is typical that land cleared for agriculture loses more than half its organic-C content.

Given that the ongoing loss of nutrients and alkalinity from soils mined by export of agricultural produce usually involves landfill or disposal to marine ecosystems with little recycling, our crude estimate of the scale of strong acid release of CO_2 is very substantial, a major part of the atmospheric increase. Substituting renewable sources of energy may have little effect in mitigating CO_2 emissions. Biological nitrogen fixation appeals as a means to significantly reduce this source of CO_2 emission. Given that CO_2 from fossil fuels is emitted on land raising regional pCO_2 and rates of photosynthesis and landscape greening, it is possible that their contribution to increased CO_2 in the atmosphere may have been overestimated.



This presentation will discuss the significance of these CO_2 emissions from soil acidification and their possible mitigation by recycling of wastes and other means. It will also propose several increasing roles for nitrogen fixation for future ecosystem sustainability.

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SESSION 3 – LEGUME NODULATION AND FUNCTION I

Chair: Associate Professor Penny Smith, La Trobe University

Interactions of nodulation with nematode parasitism

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Legumes evolved nitrogen fixing symbioses with rhizobia approximately 100 MYA, much later than interactions with pathogens and parasites. Several of the genes known to be necessary for nodulation have likely been modified from more ancient interactions with fungi and nematodes. This begs the question whether becoming a host to rhizobial symbionts has any trade-offs for dealing with pathogens and parasites, and if so, how?

The relationship of legumes with rhizobia, which form root nodules, and plant parasitic root knot nematodes, which form feeding sites called galls, shares several similarities. Both of these root organs are strong carbon sinks, but nodules usually provide fixed nitrogen while galls are exclusively parasitic. A first shared aspect of both interactions are the early signalling interactions in the rhizosphere that are necessary for partner recognition and attraction, mediated by plant exudates. One class of exudates plays a dual role in both interactions, these are flavonoids, ubiquitous plant signals with diverse structure-specific functions. A broad range of plants produce specific flavonoids as phytoalexins, as well as motility inhibitors against parasitic nematodes, while in legumes, certain flavonoids act as nod gene inducers and inhibitors. Genetic modification of the flavonoid pathway towards nematode motility-inhibiting metabolites in the model legume, *Medicago truncatula*, was able to protect plants from parasite (and fungal pathogen) infection while improving nodulation. At the infection stage, entry of rhizobia is controlled by several nodulation genes, none of which were necessary for nematode infection. However, loss of functional nodulation genes prevented a protective effect of rhizobia against successful nematode infection. This suggests that despite many similarities between these symbiotic and parasitic interactions, legumes evolved specific pathways for symbiotic interactions, and that their acquisition has not let to susceptibility for parasitic nematodes *per se*. However, legume genotypes differing in the efficiency of symbioses do show variable susceptibility to parasitic nematodes in an environment- and symbiont-specific way.

New methods for confocal imaging of infection threads in crop and model legumes

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Key words

Infection, infect thread, microscopy

Abstract

The formation of infection threads in the symbiotic infection of rhizobia in legumes is a unique, fascinating, and poorly understood process. Infection threads are tubes of cell wall material that transport rhizobia from root hair cells to developing nodules. They form in a type of reverse tip-growth from an inversion of the root hair cell wall, but the mechanism driving this growth is unknown. High resolution, 3-dimensional imaging of infection threads, and cell wall component specific labelling would greatly aid in our understanding of the nature and development of these structures. To date, such imaging has not been fully utilised, with infection threads typically imaged by bright field, by GFP-tagged rhizobia, and histochemically in thin sections. We have developed new methods of imaging infection threads using novel and traditional cell wall fluorescent labels, and laser confocal scanning microscopy. We have applied the use of a new highly intense and stable periodic Schiff rhodamine-123 label (Rae et al. 2020) (Figure 1), which has allowed for 3D imaging of infection threads in high detail. Through novel combination of the above method and calcofluor-white staining, we have also succeeded in differentially labelling infection threads, and have visualized infection thread walls and matrices separately (Figure 2). This differential labelling of infection threads and imaging of whole samples by confocal microscopy has made possible a more detailed and accurate quantification of thread phenotypes and numbers than had until now been practicable. Our methods have made the imaging and study of infection threads more efficient and informative, and present exciting new opportunities for future research in the area.

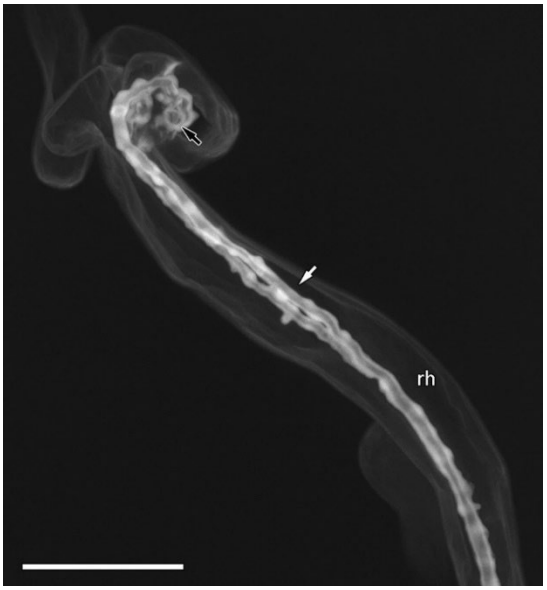

Figure 1. Infection thread after rhodamine-123 PAS labelling. Mature infection thread (white arrow) in *M. truncatula* root hair imaged with a 63× objective using a Leica SP8 confocal microscope. Maximum intensity z-projection from a 21.02 µm confocal z-stack. Scale bar equals 25 µm. Black arrow indicates position of infection chamber. *rh* root hair. From Rae (2021).

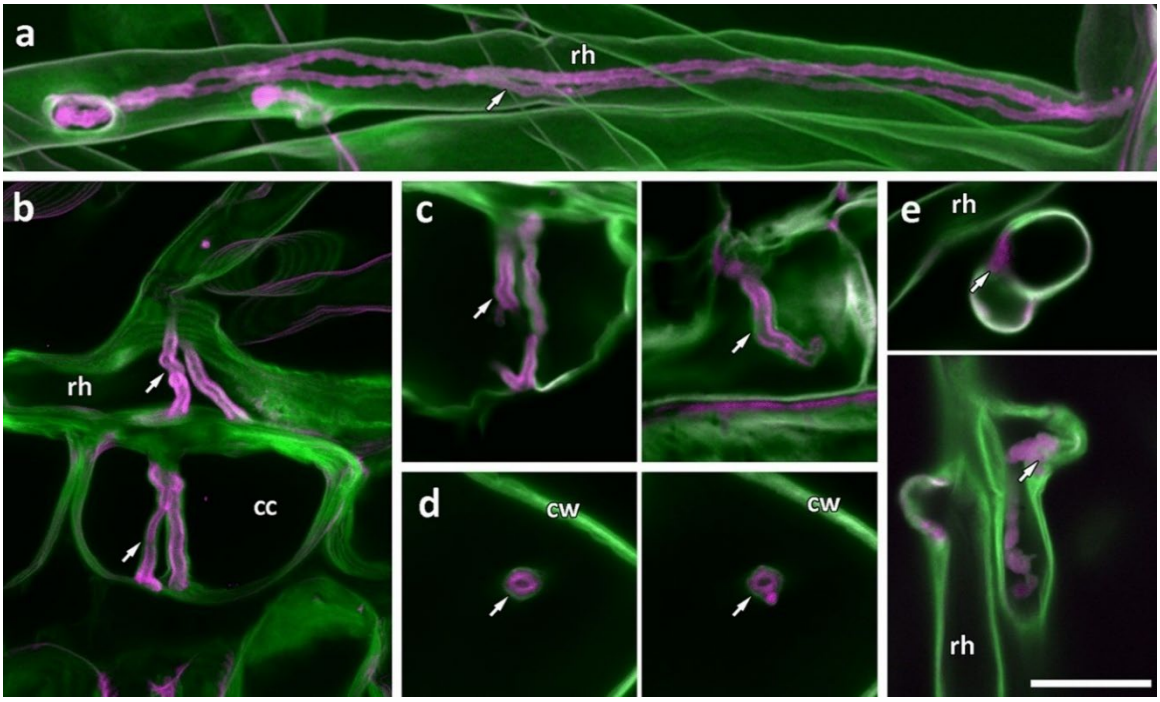

Figure 2. PAS rhodamine-123 counterstained with calcofluor white differentially labels, and shows distinct layers in infection threads. *M. truncatula* root hairs stained with PAS rhodamine-123 (magenta) then with calcofluor white (green). a) Root hair infection threads (arrow) inside a root hair are stained with rhodamine-123 whereas the dominant stain in cell walls is calcofluor white. b) to d) Cortical infection threads show two distinct layers, an outer layer stained with calcofluor white, and an inner layer stained with rhodamine-123. In (b) two infection threads exit the root hair to traverse the subtending epidermal cell and emerge in the cortical cells below. c) Infection threads in root cortical cells. d) Optical cross sections of infection threads in root cortical cells. e) Infection chambers are also differentially labelled with rhodamine-123 (arrows). Images a and b are maximum projections of 60.01 µm, and 9.99 µm confocal z-stacks. All images were captured using a 63× objective. Scale bars = 25 µm. rh root hair, cc cortical cell, cw cell wall. From Rae (2021).

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Genome-wide identification of colonisation determinants in *Rhizobium leguminosarum* using random barcode transposon-site sequencing

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Key Words
Colonisation, rhizosphere, RB-TnSeq

Abstract
Plant growth promoting bacteria must persist in the rhizosphere and colonise the roots of their host plant in order to exert their beneficial effects. *Rhizobium*-legume symbioses are one of the best characterised beneficial interactions due to their potential to alleviate our reliance on nitrogen fertilisers. Despite this, most studies on *Rhizobium* spp. have focused on its symbiotic lifestyle as an endosymbiont in root nodules and therefore the initial stages of rhizosphere growth and root colonisation remain relatively under characterised (Poole et al., 2018). The multifactorial nature of bacterial growth in the rhizosphere and root colonisation means that transposon insertion sequencing techniques such as random-barcode transposon-site sequencing (RB-TnSeq) provide a unique opportunity to study gene function at the whole genome level (Knights et al., 2021). Through incorporation of a random DNA barcode into transposons RB-TnSeq can be used to assay mutant fitness in a high throughput manner (Wetmore et al., 2015).

In this work RB-TnSeq was used in the model rhizobial strain, *Rhizobium leguminosarum* bv. *viciae* 3841, to characterise how plant species and bacterial competition affect growth in the rhizosphere and colonisation of host legumes, a non-host legume and a non-legume. A core set of 73 genes were required for growth in all plant rhizospheres and a further 58 genes commonly contributed to root colonisation. Analysis of these genes revealed that rhizosphere growth and root colonisation required the synthesis of compounds (amino acids, ribonucleotides and cofactors), alteration of metabolic function, adaptation to various stresses (such as changes in osmolarity) and sensing of external stimuli coupled with modification of gene expression. Additionally, chemotaxis and flagella-mediated motility were common prerequisites for root colonisation. Notably, many genes showed plant-specific dependencies highlighting the significant adaption required to different plant rhizospheres. This work provides a greater understanding of factors promoting rhizosphere fitness and root colonisation in plant-beneficial bacteria.

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Stable, broad host-range fluorescent markers for tracking multiple bacteria on plant roots

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Key words
Root colonisation, SynCom, fluorescence labelling

Abstract
Plant roots are colonised by a vast diversity of microorganism, with proteobacteria and actinobacteria the most important groups (Lundberg et al., 2012). Microorganisms colonise different niches within roots and alter plant fitness, are crucial in nutrient cycling, promote plant growth, prime plant defences and control pathogens (Berendsen et al., 2012). Likewise, plants shape the root microbiome, which in turn alters plant growth. Recently there has been an explosion in microbiome studies on plants. Most of them have analysed the microbiome composition by amplicon or genome sequencing (de Tkacz, 2015; Souza et al., 2016). However, the cutting-edge challenge is to move beyond counting and classifying microbiomes to understand how a microbiome assembles. One of the principal challenges in studying microbiome assembly is the identification and quantification of different bacteria during colonisation. Nevertheless, due to the huge number of different microorganisms, this has proved technically and logistically difficult. A key strategy is to establish a simpler Synthetic Community (SynCom).

We have developed a Differential Fluorescent Marker (DFM) tool which allows us to quantify and differentiate up to six bacteria in a SynCom on plant roots. The DFM tool is based on the use of three fluorescent proteins (mTag BFP, sYFP2 and mCherry), assembled in single or doble combination. The DFM plasmids are mini-Tn*7* transposon compatible with Golden Gate assembly, which integrates in the chromosome reducing fitness effect and increasing stability of the fluorescence expression cassette. Since the integration site of mini-Tn*7* is highly conserved among bacteria (Choi et al., 2005), the DFM tool is therefore broad-range. Strains labelled with the DFM tool can be quantified and differentiate with flow cytometry, which makes it independent of sequencing or culturing, and therefore less expensive and labour-intensive.

We have applied DFM tool to a synthetic SynCom (OxCom6) formed by well-known root colonisers of alpha (*Ochrobactrum pituitosum* AA2, *Rhizobium leguminosarum* 3841), beta (*Azoarcus olearius* DQS-4, *Achromobacter xylosoxydans* AT1) and gamma-proteobacteria (*Enterobacter cloacae* AA4, *Pseudomonas fluorescence* SBW25), and study their assembly on pea and barley roots. After seven days we observed a different SynCom assembly on each plant, where *P. fluorescence* SBW25 is the main coloniser on pea roots, whereas *E. cloacae* AA4 is on barley.

Our results demonstrate that DFM tool is excellent to track a highly diverse SynCom of six members on roots. However, it can be applied to other type of communities formed by different species, strains or mutants. Apart for colonisation experiments, it can be used for free-living or nodulation assays. The DFM tool uses constitutive promoters to express the different fluorescent proteins, but they can easily replace using Golden Gate.

The DFM tool is a highly versatile and modular resource to track up to six different bacteria.

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SESSION 4 – LEGUME NODULATION AND FUNCTION II

Chair: Professor David Day, Flinders University

Molecular mechanisms of legume nodulation control

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Abstract

We use genetic, physiological and functional-genomic approaches to elucidate the mechanisms driving the development and regulation of legume nodules. The host plant tightly regulates the number of nodules it forms following rhizobia-inoculation (autoregulation of nodulation) or nitrate-treatment (nitrogen-regulation of nodulation). In soybean, both processes commence with the production of novel root-derived signals, called CLAVATA3/ESR related (CLE) peptides. The genes encoding these peptides exhibit increased expression following rhizobia inoculation (*GmRIC1* and *GmRIC2*) or nitrate treatment (*GmNIC1*). Over-expression of these genes significantly reduces nodule numbers. We established that CLE peptides often require post-translational modification with a triarabinose building block to exert their full activity. The rhizobia-induced CLE peptides act systemically through the shoot, whereas the nitrate-induced CLE peptide appear to act locally in the root. Interestingly, all three CLE peptides are perceived by the same LRR receptor kinase, called Nodulation Autoregulation Receptor Kinase (NARK), which likely acts in a complex with other factors. Perception of the nodulation-suppressive CLE peptides in the shoot leads to the regulation of miR2111, a microRNA that is transported to the root where it targets the mRNA of Too Much Love for degradation to control further nodule organogenesis. In addition to the nodulation-suppression CLE peptides, the complete CLE peptide-encoding gene families of soybean (85 genes), common bean (46 genes), *Medicago truncatula* (52), and *Lotus japonicus* (53) were identified and categorised, providing a platform to help functionally-characterise these critical developmental factors. Findings relating to our progress in identifying and characterising the abovementioned nodulation factors and novel CLE peptides will be presented.

The identification of flavonoids that modulate polar auxin transport during root nodule development in *Medicago truncatula*

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Key words
Flavonoids, auxin, cytokinin, nodule development

Abstract
Rhizobia can initiate nodule in legumes, a process that resembles, but is distinct from, lateral root formation. Like lateral roots, nodules are initiated in cells of the pericycle and cortex that accumulate auxin. However, while lateral roots are inhibited by cytokinin, cytokinin signaling and synthesis is activated during nodule development. Cytokinins activate the synthesis of flavonoids inside the root, and both events are necessary for nodule initiation. Thus, this pathway appears to be specific to nodule development, and while flavonoids are necessary for nodulation, they are dispensable for other known developmental processes. During nodule development, flavonoids act as modulators of polar auxin transport, and this requires cytokinin signaling. Previous studies have shown that silencing the flavonoid pathway in *Medicago truncatula* prevented the modulation of auxin transport necessary for nodule formation. However, as plant roots can produce thousands of different flavonoids, the identity and structure of the flavonoid(s) which is required for auxin transport inhibition in legumes is currently unknown, and their identification is the aim of this project. Mass spectrometry was used to identify flavonoids induced during nodule development. Using auxin transport assays, several of these flavonoid candidates were found to be significant auxin transport inhibitors in the roots of *M. truncatula,* but the structures and identities of these flavonoids are not consistent with previous studies conducted on zucchini hypocotyls and *Arabidopsis thaliana.* Furthermore, rescue assays conducted on flavonoid deficient roots have revealed that not all of the auxin transport inhibiting flavonoids could restore nodulation in flavonoid deficient plants. This indicates that the flavonoid(s) that are responsible for auxin transport modulation during nodule formation are highly specific for this role.

Mechanisms of bacterial primary attachment to plant roots under differing pH conditions

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Key words
Attachment, pH, basic

Abstract
The initial stages of primary symbiotic bacterial attachment to plant roots are poorly characterised compared to later stages of symbiosis such as root nodule formation and nitrogenase activity (Poole, Ramachandran and Terpolilli, 2018). This study aims to characterise key mechanisms of *Rhizobium leguminosarum* bv. *viciae* attachment to *Pisum sativum* roots under differing conditions of pH.

Attachment at acidic, basic, and neutral pH was investigated by the stepwise interrogation of the repressor *praR* regulatory system using RNA sequencing, gene promoter reporter fusion assays, and *lux*-based bioreporter attachment assays of mutants. This system contains many of the genes essential for primary attachment and is differently regulated according to pH and population density (Edwards et al*.,* 2009; Frederix et al*.,* 2014; Parsons, 2019). Results show mutation of the *praR* gene causes a 79% increase in primary root attachment and a 163% increase in root colonisation 7 days post inoculation at neutral pH.

Of particular interest is the gene RL0149 which is repressed by *praR* at acidic, neutral, and basic pH and shares 55% similarity and 38% identity with *praR*. Results show that the most strongly upregulated genes in the *praR* mutant at pH 7 (> 10-fold upregulated) are > 10-fold downregulated in the RL0149 mutant in basic conditions. This pH dependent, opposite expression pattern suggests that these two XRE family transcriptional regulators may cross-regulate mechanisms of primary attachment depending on pH. Genes regulated in a pH-dependent manner in this system include the putative autoaggregation proteins *rapA2, rapB,* and *rapC,* the cadherin domain-containing calcium-binding glycoproteins *cadA* and *cadB,* and other transmembrane and exported proteins. It has been proposed that an extracellular calcium-binding protein termed rhicadhesin is a dominant mediator of rhizobial attachment via an unknown plant receptor under basic conditions (Smit, Kijne, and Lugtenberg, 1987). However, neither the protein nor its gene have been isolated or identified. The effect observed by the hypothetical rhicadhesin may be the combined effect of adhesins cross-regulated by the *praR*-RL0149 system.

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A master regulator of primary attachment to pea roots

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Key words
Symbiosis, pea roots, bacterial attachment, ECF, transcription

Abstract
Bacterial attachment to plant roots, the first step in bacteria-legume symbiosis, is an important factor in microbial competitiveness. Elucidation of the role of genes involved will deepen understanding of the steps leading to effective nitrogen fixation, including microbial colonisation of roots.

We sought to understand the involvement of a three-gene operon (RL3234-6) in initial attachment of *Rhizobium leguminosarum* to pea roots. Expression of RL3234 (encoding lipoprotein LppE) is up-regulated up to 100-fold in the pea rhizosphere and approx. 6-fold by the addition of phenylalanine (Phe) to media (Ramachandran et al., 2011). Following inoculation onto pea roots, *lppE* has been shown to have a specific spatial expression pattern by monitoring with a Lux reporter fused to the *lppE* promoter. Other genes in the operon encode an extracytoplasmic sigma factor (ECF) EcfE (RL3235) and anti-extracytoplasmic sigma factor (ASF) AsfE (RL3236). In general, ECF and ASF proteins play a role in regulating transcription in response to extracellular signals.

Results

1. A role for the *lppE* operon in attachment was investigated using attachment assays of 1 hr duration to six-day-old *Pisum sativum* roots. Roots were submerged in cell suspensions of Rlv3841 wild type, *ΔlppE, ΔecfE* and *ΩasfE* strains labelled with a constitutively expressed Lux reporter plasmid. After imaging, luminescence values were used to calculate the number of bacteria attached. Mutants in *lppE* and *asfE* showed significantly higher attachment to pea roots than wild type, showing their mutation affects attachment.
2. Transcriptional regulation of the operon by its own gene products was investigated using the fact that Phe induces expression. The spatial and temporal pattern of Phe on pea roots has been previously demonstrated (Pini et al., 2017). To follow expression from the *lppE* promoter, a Lux reporter fused to the *lppE* promoter was introduced into Rlv3841 wild type, *ΔlppE, ΔecfE* and *ΩasfE* strains. Expression was significantly down-regulated in *ΔecfE* compared to wild type, while significantly up-regulated in *ΩasfE*. This shows that transcriptional control of LppE expression is exerted by both EcfE and AsfE. This finding was verified *in planta* with strains inoculated onto *P. sativum* plants and imaged and luminescence measured 6 days post inoculation.
3. LppE does not transcriptionally regulate the operon since there is no significant difference in the activity of the *lppE* promoter in Rlv3841 wild type and *ΔlppE* backgrounds. However, *lppE* transcriptional and translational fusions to a GusA reporter were used to investigate a

translational regulatory role. Results show overexpression of LppE inhibits translation of the operon.

4. Ground *P. sativum* root induces *lppE* promoter activity, shown using the Lux reporter fused to the *lppE* promoter in Rlv3841 wild type grown in liquid culture supplemented with ground root. Treating root grindate with proteinase K or viscozyme L prevent this induction, suggesting that the *lppE* promoter may become active in response to a root glycoprotein.

Conclusions

Root attachment is the primary determinant of competitive success in *R. leguminosarum*, and the first step in forming a highly specific symbiosis between nitrogen-fixing bacteria and their legume hosts. We have investigated the role of RL3234-6 (*lppE ecfE asfE*) operon in *R. leguminosarum* attachment to pea roots:

- LppE and AsfE play a role in bacterial attachment, possibly preventing hyper- or non-specific attachment.
- EcfE activates transcription from the *lppE* promoter, while AsfE represses it.
- LppE is a translational repressor of the operon.
- Induction of transcription is seen in response to a glycoprotein present in pea roots.

The operon's role in root attachment is tightly regulated, and most likely responses to extracellular signal(s) from the plant root interacting with LppE in the periplasm with the signal transmitted via membrane-localised AsfE.

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SESSION 5 – PHYSIOLOGY & GENETICS OF SYMBIOSIS I

Chair: Dr Jason Terpolilli, Murdoch University

Adaptation, selection and sanctioning in the *Rhizobium*-legume symbioses

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Abstract

Colonization by bacteria of the zone surrounding plant roots (rhizosphere) is crucial to plant productivity, with plants secreting 10-30% of total photosynthate to the rhizosphere. Microarray, RNAseq and metabolic analysis combined with InSeq analysis of growth in the rhizosphere, colonization of roots, bacteroid formation and regrowth from nodules has been used to dissect the stages in root colonisation and N_2-fixation by *Rhizobium leguminosarum* in its interaction with pea. During infection of legumes the metabolic repertoire of rhizobia is dramatically restricted with a dramatic reduction in metabolic diversity in mature bacteroids. While InSeq analysis allowed identification of virtually all the known *nif* and *fix* genes needed by rhizobia inoculated as single inocula on pea, it revealed a much larger set of genes essential for competitiveness for root colonization and formation of N_2-fixing bacteroids (1). By comparing Inseq experiments for rhizosphere growth, pea root attachment, bacteroid formation and regrowth of rhizobia from nodules (i.e. pre-differentiation rhizobia in infection threads) we have constructed the first comprehensive map of the genes needed for the lifestyle changes of *R. leguminosarum.* Furthermore, to understand how N_2-fixation is controlled and the metabolic basis for ammonia secretion by bacteroids, we used genome scale modelling, flux balance analysis and experimental metabolic flux determinations to show how plants control bacteroid metabolism and promote ammonia secretion (2). We went on to develop high throughput methods to screen rhizobia and competitiveness and effectiveness as these are key determinants of the success and yield of legumes (3). This led us to consider how legumes ensure mutualism by sanctioning cheating bacteria while favouring efficient N_2-fixing rhizobia (4).

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Iron transport in nodules – some key players identified but questions remain

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Key words

Iron, symbiosome membrane, VTL, YSL

Abstract

Iron is essential for symbiotic nitrogen fixation. It is required by the plant for the synthesis of leghemoglobin and in the bacteroid it is important for synthesis of nitrogenase, ferrodoxin, and enzymes of the respiratory chain. This means that the nodule is a major sink for iron. Iron is imported into the nodule via the xylem most likely as ferric citrate. To reach the bacteroid it must cross a number of cell layers, be imported into the infected cells and then across the symbiosome membrane (SM) (Brear et al. 2013). In our proteomes of the soybean SM (Clarke et al. 2015; Brear et al. 2020) we identified three proteins that have homology to known metal transporters. These include two vacuolar iron transporter-like (VTL) proteins, GmVTL1a and GmVTL1b, and a Yellow-Stripe-Like7 transporter, GmYSL7.

GmVTL1a and GmVTL1b are members of the vacuolar iron transporter (VIT) family which includes *Arabidopsis* AtVIT1 and yeast CCC1 that transport ferrous iron into the vacuole. Both genes have high expression in nodules and promoter-GUS fusions show that their expression is concentrated in infected cells (Liu et al. 2020; Brear et al. 2020). When expression of GmVTL1a and 1b is knocked out nodule development is impaired and nitrogen fixation reduced (Liu et al. 2020) and the same effect was seen in mutants for the *Lotus japonicus* orthologue of *GmVTL1*, *LjSEN1* (Hakoyama et al. 2012). We have shown that VTL1a but not VTL1b or LjSEN1 can complement the yeast vacuolar iron transport mutant *Δccc1* suggesting it functions as a ferrous iron transporter. Although SEN1 cannot complement *Δccc1* yeast, expression of GmVTL1a in the *sen1* mutant restores nodule structure and nitrogen fixation. These results suggest the VTL1a is responsible for iron transport into the symbiosome and that SEN1 is an iron transporter *in planta*. VTL1b (but not SEN1) can restore growth of a yeast mutant in vacuolar manganese transport suggesting that it may be a manganese transporter.

GmYSL7 is a member of the YSL family of transporters, part of the wider oligopeptide transporter (OPT) family. Most YSL proteins transport metal-phytosiderophore complexes and are important for plant metal homeostasis (Brear et al. 2013). However, the *Arabidopsis* orthologue AtYSL7 is responsible for the import of a peptide derivative, Syringolin A, produced by the bacterial pathogen *Pseudomonas syringae,* into the cytoplasm of plant cells, suggesting it is a peptide transporter (Hofstetter et al. 2013).

We showed that both AtYSL7 and GmYSL7 transport oligopeptides in yeast and that silencing GmYSL7 in infected cells results in nodules with reduced nitrogenase activity and defects in symbiosome development (Gavrin et al. 2021). We could not show transport of Fe(II)-NA in a yeast iron transport mutant. However, another study has suggested that GmYSL7 is a low affinity iron-NA transporter and that the transcript is also present in the nodule cortex, where it mediates iron import into nodule cells, but that it is also present on the SM (Wu et al., 2022). Our RNAseq data for YSL7-silenced nodules suggests a link for GmYSL7 to iron homeostasis (Gavrin et al. 2021). However, the proposed direction of transport on the SM is out of the symbiosome into the cytosol down the electrochemical gradient, raising the question of whether GmYSL7 has two functions in nodules, as an importer of iron into cells and importing iron or peptides out of the symbiosome. The implications of this for nodule function and regulation of iron homeostasis will be discussed.

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Genetic determinants of host range in *Mesorhizobium ciceri* strains WSM1271, WSM1497 and WSM1284

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Key words
Nodulation, *Mesorhizobium*, *Biserrula, Lotus, Ornithopus*

Abstract
N_2-fixing symbioses between rhizobia and legumes are established following an exchange of chemical signals and results in rhizobia infecting legume roots. However, not all interactions are compatible as there can be a very high level of specificity between legumes and strains of rhizobia. This level of specificity determines the host range of a particular strain of rhizobia, with narrow range strains only nodulating one or a few legume species, while broad host range strains may nodulate across multiple legume genera. A key determinant of host range is rhizobial Nod factor, the synthesis and secretion of which is encoded by rhizobial nodulation (*nod*, *noe*, *nol*) genes. While the genetic basis of host range differences between strains has been investigated for several decades, very few studies focussed on the rhizobia in *Mesorhizobium* genus.

Biserrula pelecinus is an annual pasture legume that forms an effective symbiosis with strains of *Mesorhizobium* spp. and provides a hard-seeded pasture, tolerant to grazing in mixed farming systems. Since its introduction, *B. pelecinus* has been successful in producing high yields under severe drought or limited rainfall in regions of New South Wales and Western Australia. Both the commercial inoculant strain*, M. ciceri* WSM1497 (Brewer et al*.,* 2017) and *M. ciceri* WSM1271 (Nandesena et al., 2013) only effectively nodulate this host. By contrast, *M. ciceri* WSM1284 (Haskett et al*.,* 2016) has a broader host range, nodulating species across eight genera: *Antopetitia, Astragalus*, *Biserrula*, *Glycyrrhiza*, *Leucaena, Lotus, Ornithopus* and *Scorpiurus*. The genetic cause/s for these differences in host specificity is unknown.

Interrogation of the WSM1271, WSM1497 and WSM1284 genomes revealed different sequences and content of genes required for regulation and synthesis of Nod Factor. While WSM1284 has 21 nodulation genes, WSM1271 and WSM1497 have only 15 and 14 nodulation genes, respectively. Six of the additional WSM1284 genes are appear to code for fucose biosynthesis (*noeL*, *nolK*, *noeJ*, *noeK*), transfer (*nodZ*) and acetylation (*nolL*). While all three strains harbour multiple copies of *nodD* and *nodA*, the sequences between the broad and narrow host range strains diverge, indicating the NodDs may respond to different flavonoids, and that Nod factors may vary in length and saturation of the acyl tail.

Putative nodulation genes encoding transcriptional regulators (encoded by *nodD*) and genes involved in Nod factor synthesis (encoded by *nodA*, *nodZ* and *nolL*) were targeted and 14 mutants generated. The phenotype of these strains was evaluated in symbiosis with *B. pelecinus, Lotus ornithopodioides* and *Ornithopus sativus*. Three mutants (Δ*nodD1*::*nptII,* Δ*nodZ*::*nptII,* Δ*nolL*::*nptII*) differed in their ability to nodulate all three hosts compared to WSM1284 (Table 1). Despite, *nodD1* and *nodD2*

functionally complementing one another on *L. ornithopodioides*, *nodD1* was essential for nodulation of *B. pelecinus* and *O. sativus*. Deletion of *nodZ* abolished nodulation in *O. sativus*, whereas deletion of *nolL* abolished nodulation in *O. sativus* and *B. pelecinus*. The symbiotic phenotypes suggest these genes are involved in WSM1284 having a broad host range. While deletion of some nodulation genes restricts host range of WSM1284, the modified host ranges differ to WSM1271 and WSM1497. Therefore, a combination of nodulation genes and other host range determinants may be responsible for WSM1284 broad host range. This study will assist with understanding the genetic determinants of host range in *Mesorhizobium* strains.

Table 1. Nodulation phenotypes of *B. pelecinus*, *L. ornithopodioides* and *O. sativus* inoculated with five *Mesorhizobium ciceri* WSM1284 mutants. Nodules present (+), nodules absent (-).

WSM1284 Genotype	**Gene Function**	***B. pelecinus***	***L. ornithopodioides***	***O. sativus***
Wild-type		+	+	+
Δ*nodD1*::*nptII*	Transcriptional	-	+	-
Δ*nodD2*::*nptII*	regulator	+	+	+
Δ*nodD1*::*nptII,*Δ*nodD2*		-	-	-
Δ*nodZ*::*nptII*	Fucose moiety	+	+	-
Δ*nolL*::*nptII*	Acetylation of Fucose	-	+	-

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Polyamines are essential for bacteroid maintenance and N_2-fixation

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Key words
Polyamines, bacteroid, senescence

Abstract
Polyamines are ubiquitous positively-charged molecules involved in a variety of physiological processes in all domains of life. The most basic polyamines are diamines such as cadaverine (1,5-pentanediamine) or putrescine (1,4-butanediamine), which are derived from amino acid catabolism. Putrescine is the precursor for more complex polyamines such as homospermidine (N-(4-aminobutyl)-1,4-butanediamine) or spermidine (N-(3-aminopropyl)-1,4-butane-diamine). Detailed studies in *Escherichia coli* have highlighted the involvement of polyamines in multiple and diverse processes. Most notably, polyamines are involved in ribosome (30S subunit) assembly and translational activity. In addition, they are involved in mediation of oxidative, osmotic, and acidic stress (for review see Igarashi and Kashiwagi 2018).

Although comprehensive knowledge about the exact mechanisms involved is still missing, polyamines are also important in host – pathogen associations, including in animal- and plant-pathogenic bacteria (Sha and Swiatlo 2008; Gerlin, Baroukh and Genin 2021). However, comparatively little is known about the importance of polyamines in rhizobia – legumes symbioses (Hidalgo-Castellanos et al. 2022), with one mutant study reporting a reduction in nodulation efficiency on alfalfa in a polyamine-lacking *Sinorhizobium meliloti* strain (Becerra-Rivera et al. 2020).

Here, we describe the importance of "Homospermidine Synthase" (Hss) in *Rhizobium leguminosarum* bv. *viciae* 3841 and other rhizobia. This gene was characterised as essential for symbiosis in an earlier INSeq analysis (Wheatley et al. 2020). Importantly, for most rhizobia, homospermidine is the only complex polyamine produced and appears to have functions that cannot be covered by putrescine. Mutants in *hss* were reduced in growth and showed an increased susceptibility to stress such as acid shock. Moreover, in symbiosis with host plants, the mutants displayed reduced nitrogen fixation and smaller nodules. Over prolonged growth periods this resulted in stunted and chlorotic plants, indicative of nitrogen starvation. Detailed morphological analysis showed signs of premature senescence and bacteroid degradation. Heterologous complementation experiments demonstrated that the role of homospermidine in the rhizobia – legumes symbioses is not unique as it can be replaced with other complex polyamines. RNAseq analysis of the mutant revealed a drastically changed transcriptional landscape compared to the wild type. In agreement with previously reported phenotypes of polyamine-lacking mutants (see references above), we found an upregulation of genes related to stress responses, translation, and motility, whereas many transport systems were downregulated.

Overall, we show that complex polyamines are needed to maintain a normal physiology, adapt to stressful environments, and for maintaining specifically bacteroid functioning and integrity.

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Nodulin 26 is a multifunctional plant aquaporin that facilitates ammonium transport in nitrogen-fixing soybean nodules

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Key words

Symbiosome, ammonium, Nodulin 26

Abstract

The basic metabolic exchange between the plant and bacteroids during symbiotic nitrogen fixation in legumes is carbon from the plant, in the form of malate, for fixed nitrogen from the rhizobial bacteroids, in the form of ammonia/ammonium and possibly amino acids. During the symbiosis, legumes develop highly specialised organs on their roots, called nodules, which are infected by the rhizobia. Inside the infected nodule cells, the rhizobia differentiate into nitrogen-fixing bacteroids that are excluded from the plant cytosol in organelle-like structures termed symbiosomes. The symbiosome membrane acts to regulate metabolite exchange between the symbionts through transport proteins synthesised by the plant. A number of transport processes have been biochemically characterised in isolated symbiosomes, but the molecular identity of many of the transporters involved remains unknown. In particular, the transporter that facilitates efflux of fixed nitrogen from symbiosomes remains unidentified. This efflux is thought to be predominantly through a non-selective cation channel characterised in patch-clamped symbiosomes from soybean, which is inwardly rectified by cytosolic magnesium and prefers ammonium (Tyerman et al. 1995). However, no candidate channel protein has been identified from several transcriptomic and proteomic studies.

The aquaporin Nodulin 26 (NOD26) is a major protein in the soybean symbiosome membrane, and recently at least some aquaporins have been shown to transport ions as well as water (Tyerman et al. 2021). We have heterologously expressed GmNOD26 in *Xenopus laevis* oocytes and observed monovalent cation-induced currents, very similar to those reported in the previous patch clamp studies of symbiosomes. The channel is permeable to ammonium, methylammonium, potassium and sodium, but not choline or anions, and these currents are inhibited by divalent cations. Through phosphomimetics of Serine262, we have identified a switch in the permeability of NOD26 to water or cations when expressed in the *Xenopus* oocytes. We suggest that the soybean aquaporin NOD26 is a multifunctional channel that facilitates both ammonia and ammonium transport across the symbiosome membrane in nitrogen-fixing nodules, and that this transport is regulated by phosphorylation of a key serine residue.

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SESSION 6 – PHYSIOLOGY & GENETICS OF SYMBIOSIS II

Chair: Dr Joshua Ramsay, Curtin University

Relationship between central metabolism and nitrogen fixation in *Sinorhizobium meliloti*

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Keywords
Carbon metabolism, *Sinorhizobium meliloti*, nitrogen fixation

Abstract
Carbon metabolism is well understood in *Sinorhizobium meliloti*. The literature is consistent with the role dicarboxylic acid metabolism plays while the bacteroid is actively fixing nitrogen. However, the literature also contains many nitrogen fixation phenotypes ascribed to mutants that encode enzymes in central carbon metabolism that make little sense or are even paradoxical. For example, a mutation in *pckA*, which encodes phosphoenolpyruvate carboxy kinase and is necessary for gluconeogenesis, consistently gives nitrogen fixation rates that are approximately 50% of wild-type (Finan et al., 1991). Yet no measurable enzyme activity can be detected in bacteroids. Similarly, it has been shown that strains that do not have triose phosphate isomerase activity also yield plants with 50% dry matter accumulation when grown under nitrogen deficient conditions (Poysti and Oresnik, 2007). The reason why these mutations give rise to nodules that have reduced levels of nitrogen fixation is not currently understood.

Within the genome of *S. meliloti* there are three genes that are annotated as putative transketolases. These genes are *tktA* (*SMc03978*), *tktB* (*SMc02342*), and *cbbT* (*SMb20200*) which is part of the Calvin Benson Bassam operon. While screening for mutants that did not acidify their growth medium, we isolated a strain carrying a transposon insert within the gene *tktA* (Hawkins et al., 2018). When this strain was inoculated onto alfalfa it led to small, white nodules with poor structure and no evidence of bacterial invasion. However, occasionally pink nodules also formed. *S. meliloti* isolated from these nodules were found to carry a mutation in the gene *SMc02340* which encodes a transcriptional regulator. Subsequent characterization showed that SMc02340 regulated *tktB*.

To characterize the pentose phosphate pathway in *S. meliloti* a strain containing mutations in both *tktA* and *tktB* was constructed, essentially eliminating this pathway. To reimagine this metabolic pathway, a phosphoketolase from *Bifidobacterium adolescentis* was introduced to provide a metabolic bypass pathway. Although the introduction of the phosphoketolase could bypass the pentose phosphate pathway, a few sporadic colonies were found on the negative control plates for the strain carrying both the *tktA* and *tktB* mutations. Subsequent purification these colonies and whole genome sequencing identified a single mutation in the gene *cbbR*. This was shown to lead to an overexpression of the *cbb* operon including *cbbT*.

Although these suppressors that led to *tktB* or *cbbT* expression allowed normal symbiotic development, the plants only accumulated 30-50% dry weight when grown under nitrogen limiting conditions (Figure 1).

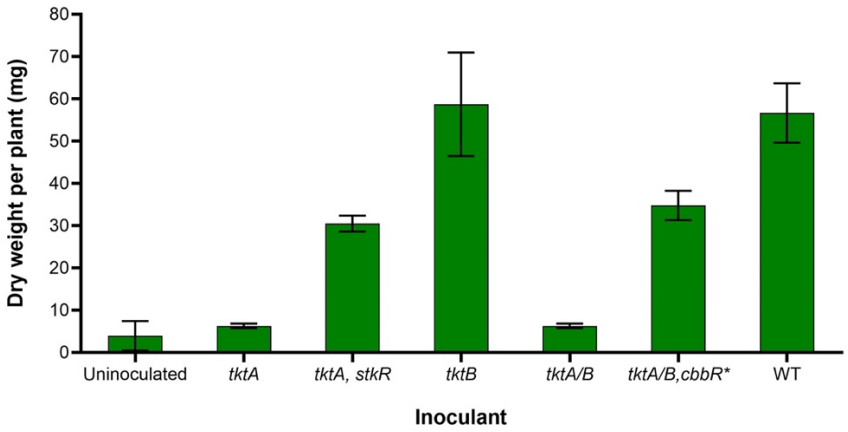

Figure 1. Dry weight production of *Medicago sativa* inoculated with a range of *S. meliloti* 1021 metabolic mutants.

Based on our current knowledge, there is no clear explanation why or how these lesions affect nitrogen fixation. Collectively, mutations that affect central carbon pathways can lead to associations with a reduced capacity to fix nitrogen. Since it is unlikely that these are directly affecting nitrogenase activity, they must affect some aspect of nodule development. We are hypothesizing that mutations that restrict carbon flow to the pentose phosphate pathway or the upper half of the Embden Meyerhoff Parnas pathway may lead to lower levels of endoreduplication, thus decreasing the gene dosage of nitrogenase and its associated components ultimately leading to lower levels of nitrogen fixation.

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The physiology, metabolism and energetics of respiratory chains in *Paraburkholderia sprentiae* WSM5005, in free-living and symbiotic conditions

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Key words
B-proteobacteria, symbiosis, transcriptomics, perennial legume

Abstract
Paraburkholderia sprentiae WSM5005 is a β-rhizobia isolated from nodules on the perennial legume, *Lebeckia ambigua*, with both bacterial and legume partners being native to the fynbos biome of the Western Cape floristic region of South (le Roux and Van Wyk, 2007; De Meyer et al., 2013). Initial investigations aimed to establish the potential for WSM5005 as a suitable N_2 fixing microsymbiont for the development and introduction of *L. ambigua* as a domesticated, perennial legume to the agricultural regions of the South West of Western Australia (Howieson et al., 2008; De Meyer, Cnockaert et al., 2013; Howieson et al., 2021). These preliminary studies described several fundamental differences in the genomic architecture of WSM5005 when contrasted to the α-rhizobia which, in addition to the chromosome, was composed of a large, secondary replicon (presumed to be a second chromosome), three plasmids, and showed a diverse arrangement of the *nif/fix* genes. These features appeared common, although not entirely conserved, when compared to other N_2-fixing β-rhizobia (De Meyer et al., 2016). Furthermore, a significant and unifying attribute of β-rhizobia was the absence of *fixNOQP* and *fixGHIS* genes, encoding cytochrome cbb_3, previously believed to be essential to N_2-fixation (Preisig et al., 1996). The findings presented a clear knowledge gap in the physiology and genomic architecture of β-rhizobia, and proposed a paradigm shift in rhizobial research. To explore and elucidate some of these foundational differences between the α-rhizobia and β-rhizobia, alongside the practical application of developing WSM5005 as a potential inoculant, this study aimed to characterise the physiology of *P. sprentiae* WSM5005 in symbiosis with *L. ambigua*, while further investigating the novel genome architecture and respiratory chains.

Bioinformatic analysis resolved a multipartite genome consisting of a primary chromosome, a chromid, and three accessory replicons (pPS1, pPS2, pPS3). Genome interrogation confirmed the absence of a cytochrome cbb_3 complex, while alternative terminal oxidases cytochrome bo_3, *-bd* (type I), and *-bd* (type II) (Borisov et al., 2011), were found distributed across the chromosome, chromid, and pPS1. Essential genes were found to be enriched on the chromosome and chromid while, except for three (out of multiple duplicates) complete or truncated *fixAB* or *fixABC* complexes, distribution of *nif* and *fix* genes were found to be restricted to the chromid and the pPS2, with most confined to a ~40Kb region, duplicated within each replicon with a 99% homology.

To probe the expression and functionality of these duplications in symbiosis, full genome transcriptomes were created for WSM5005 free-living cells and bacteroids, followed by comparative analyses of genes pertaining to N_2-fixation, central metabolism, and microaerobic respiration. All

duplications of *nif* and *fix* genes appeared to be expressed in the bacteroid. While there was an apparent bias toward the expression of genes located on the WSM5005 chromid in symbiosis, there appeared to be no continuity between the expression of *nif* genes shared across the chromid and pPS2, suggesting that while the chromid may play an integral role in symbiosis, it is not the sole replicon enriched for expression in that environment. Furthermore, while expression patterns were consistent with C_4-dicarboxylates being the primary source of carbon in the bacteroid, the induction of genes relating to central metabolism, the TCA cycle, and nitrogen metabolism are suggestive of an alternate carbon flux, likely correlated to the differing redox poise associated with the function of cytochrome *bd*, compared to cytochrome cbb_3. Mutational studies showed WSM5005 lacking a functional cytochrome *bd* continued to fix nitrogen at wildtype rates, however, free-living experiments demonstrated significant changes in survival.

Together, the results presented demonstrate WSM5005 to be an effective, N_2-fixing microsymbiont. While the functional role of chromid remains unclear, expression of the symbiotic genome shows a respiratory flexibility and redundancy, while suggesting additional routes for carbon sinking to compliment and enable this. Fully characterising the intrinsic link between carbon metabolism and the functional respiratory chains in WSM5005 bacteroids will, in turn, provide significant insight into N_2 fixation in beta-rhizobia.

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Modelling the Legume-Rhizobium Symbiosis

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Key words
Sanctioning, mixed nodules, statistical modelling

Introduction

Nitrogen fixation is an energy intensive process (Denison, 2021). We would therefore expect that within a community, such as that of the rhizosphere, bacterial strains that cannot fix nitrogen should have a fitness advantage over those that can. Therefore, for the symbiosis between legumes and nitrogen fixing bacteria to be evolutionary stable the relationship requires regulation by the plant to prevent 'cheating' bacteria exploiting the supply of carbon without providing nitrogen in return (Denison, 2000; West et al., 2002). The method of this control has been demonstrated to be conditional nodule sanctioning (Westhoek et al., 2017, 2021) in which the plant will no longer supply carbon to nodules who are providing less nitrogen relative to the global nitrogen signal. However, several questions around sanctioning remain. These include: the mechanism by which the plant can detect a nodules nitrogen output and then impose sanctions, as well as the resolution of sanctioning i.e., the level at which a plant is able to detect variations in nitrogen output. In this work we have again demonstrated sanctioning as the method of control as well as developed a statistical model of sanctioning to unravel the underlying mechanism. The model has been used here to demonstrate the plant cannot differentiate between the nitrogen output of individual cells within a nodule and instead will treat a mixed nodule based on the nodule's overall output.

Results

Using flow cytometry, sanctioning of inefficient nodules was demonstrated. *Pisum sativum* was co-inoculated with two isogenic strains of *Rhizobium leguminosarum*, differing only in their nitrogen fixing ability and fluorescent tag. Three strains were used with fixation capacities of 0%, 50% and 100%. Flow cytometry was used to measure the total number of bacteria as well as how many of the bacteria had differentiated into nitrogen fixing bacteroids. Sanctioning led to a significant decrease in the total count of bacteria within less efficient nodules as well as a significant decrease in the proportion of bacteroids in the less efficient nodule. To gain a greater understanding of how a plant sanctions inefficient nodules, a statistical model of the relationship between a nodule's fixing ability and the proportion of bacteroids within this nodule was developed (Figure 1A). The model was the best fit (models were compared via their Akaike Information Criterion score) of several models produced and was a statistically significant fit for our data.

After demonstrating conditional sanctioning of inefficient nodules, we then investigated whether the plant is capable of conditional sanctioning at the cellular level. To do this, mixed nodules (containing two strains) were analysed using flow cytometry and confocal microscopy. Flow cytometry data was collected both for individual strains (differentiated via their fluorescent tag) and for the whole nodule. When cellular data, the mean proportion of bacteroids at each relative fixation ability, was plotted in

our model, the data was a poor fit. However, the whole nodule data plotted against the average relative fixation rate of the strains fitted the model well (Figure 1B). Importantly, whole nodule data has the same trend (positive) as the model of sanctioning while cellular data forms a peak at fifty percent efficiency. Confocal microscopy was also used to demonstrate that there was no difference in the health or development of cells within mixed nodules regardless of whether they were infected with the efficient or inefficient strain (Figure 1C).

Conclusion

Flow cytometry data showed that the plant can detect the global nitrogen signal, compare the output of different nodules, and then sanction nodules that weren't supplying nitrogen as efficiently as others. Importantly this was true regardless of the strain combination. This means that the plant is not using a threshold value of nitrogen output to determine sanctioning. This data allowed us to produce a statistical model of sanctioning as it relates to relative nitrogen fixation. This model shows that as relative fixation ability increases the proportion of bacteroids, and therefore the supply of nitrogen to the strain increases. In this work we found that there was no evidence for cellular sanctioning as the strain specific data did not fit our model as well as the whole nodule data. This conclusion was supported by our confocal microscopy, in which no change to cell health or development was observed. From this it may be concluded that plants treat mixed nodules based on their overall nitrogen output. This implies that sanctioning only occurs at the nodular level.

In future work we plan to improve this model and use it to test candidate genetic pathways as well as signalling molecules for the regulation of sanctioning. These candidates will be generated based on the results of RNA sequencing to be carried out in the near future.

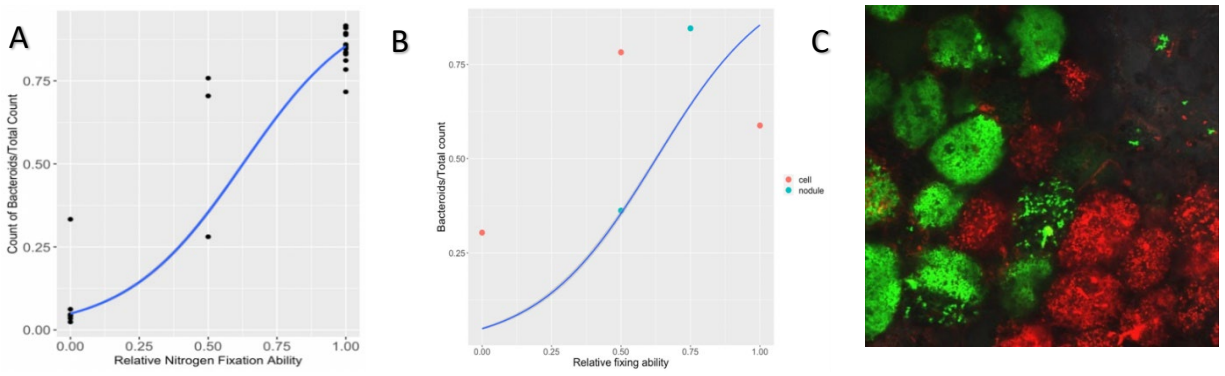

Figure 1. A generalised linear model of the relationship between relative nitrogen fixing ability and the proportion of bacteroids (a measure of sanctioning) was produced (A). This model demonstrates that as relative fixing ability increases so does the proportion of bacteroids. Therefore, as fixing ability decreases the level of sanctioning increases. Cellular sanctioning was then tested by plotting mixed nodule data over the model (B). As shown, the whole nodule data fits our model much better than the cell specific data suggesting cellular sanctioning is not occurring. This conclusion was also supported by our confocal microscopy data in which we saw no difference in the health or development within mixed nodules between the cells infected with efficient (red) and inefficient (green) strains of *R. leguminosarum* (C).

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Tracking N_2-fixation-efficiency effects on plant and nodule growth

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Key words

Non-destructive assay, hydrogen, N-per-C, transpiration

Abstract

N-per-C efficiency differs among legume hosts (Oono and Denison 2010) and rhizobia strains (Oono et al. 2020) but neither this ratio nor its effects on the rate or timing of plant and nodule growth are widely measured. We will discuss improvements in our published efficiency assay, plus new non-destructive methods to track plant and nodule growth.

Non-destructive assay for N_2-Fixation

Quantifying N_2-fixation rates and N_2-fixation efficiency is critical for research to reduce environmental and economic costs of nitrogen fertilizers. Improving N_2-fixation efficiency could prevent plants from investing too much carbon in nodules, which could decrease yields. N content and ^{15}N ratio at harvest capture only a snapshot of fixed nitrogen. While non-destructive assays like acetylene reduction can be used to measure responses to fluctuating environmental factors such as light and drought, acetylene is explosive and triggers decreases in nodule activity.

The N-per-CO_2 efficiency of rhizobia strains is the product of two parameters: the Electron Allocation Coefficient (EAC, fraction of nitrogenase activity making ammonia rather than hydrogen) and the ratio of nitrogenase activity to nodule interior respiration. Modified Magenta units or growth pouches are used as flow-through gas exchange cuvettes (Oono and Denison 2010; Oono et al. 2020). Gas-flow through each chamber is a mix of O_2 and either N_2 or Ar. Hydrogen gas produced by nitrogenase is measured using an electrochemical sensor, and root-plus-soil respiration is measured as CO_2 production using CO_2 analysers. Small decreases in O_2 are assumed to reduce the nodule-interior respiration that supports N_2 fixation, whereas root and soil respiration are oxygen-saturated (Witty et al. 1983). The N_2- fixation efficiency (ratio of the change in H_2 production to the change in respiration) can be multiplied by the EAC (measured by switching to ArO_2) and then by 2/3, based on the relative electron requirements per mole of NH_3 vs. H_2.

Water Uptake Monitoring

Measuring plant water uptake throughout an experiment is a useful proxy for leaf area per plant. This technique allows quantification of plant growth responses to contrasting rhizobia strains (Figure 1). By monitoring plant growth over time, rather than at a single time point at the end of an experiment, we can observe whether differences in growth occur when plants start fixing nitrogen, and whether those differences correspond to sanctions on non-fixing nodules.

Nodule Growth Monitoring

Assessing nodules at harvest gives useful data on one time-point, including nodule size and location, but measuring nodule development over the course of an experiment offers greater insight on specific rhizobia behaviour. Growth pouches provide an excellent opportunity to track nodules over time. Artificial intelligence, such as RootPainter software (Smith et al. 2022), can be used to identify and measure nodules in a high-throughput manner. These methods can track differences in nodule formation and growth rate among nodules with different strains (Figure 2).

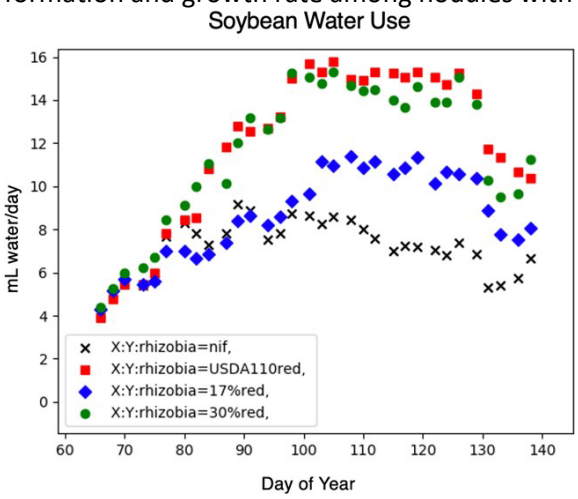

Figure 1. Water use per day, a proxy for leaf area, in soybean plants inoculated with mixtures of fixing (USDA110red) and nonfixing (nif) rhizobia. Plants with 30% or more of the fixing strain grew faster than those with 17% or fewer of the fixing strain.

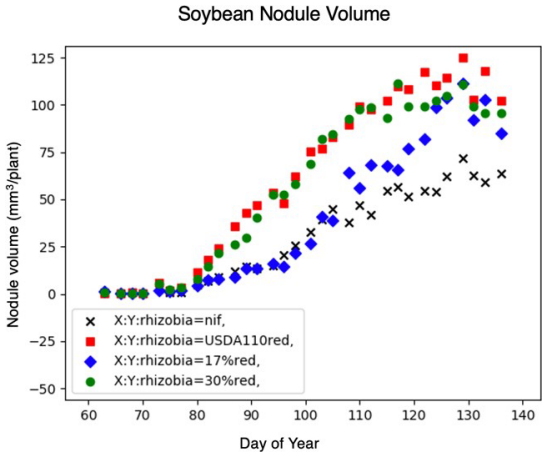

Figure 2. Total nodule volume in soybean plants inoculated with mixtures of fixing (USDA110red) and nonfixing (nif) rhizobia. Nodule volume estimated by Root Painter based on diameter measurements and assumption of spherical nodules.

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SESSION 7 – EVOLUTION AND DIVERSITY OF RHIZOBIA I

Chair: Dr Liz Farquharson, South Australian Research & Development Institute (SARDI)

The role of root nodule bacteria in the recovery of *Sophora toromiro*: The extinct Easter Island legume tree

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Key words

Mesorhizobium, restoration, Rapa Nui

Abstract

Rapa Nui (Easter Island) is in the Micronesia-Polynesian hotspot which comprises some 4,500 islands in the South Pacific and is considered an epicenter of global biodiversity loss. *Sophora toromiro* (Phil.) Skottsb. is a legume tree endemic of Easter Island that is currently categorized as extinct in the wild since the last specimen was cut in the late 1950's. This species is described as a small tree highly valued for its wood. It belongs to the Edwardsia section of the *Sophora* genus that groups approximately 20 species distributed in the southern hemisphere (Peña et al. 2000; Mitchell and Heenan, 2003).

Existing germplasm of *S. toromiro* is reproduced and sheltered at Botanical Gardens mainly in Europe. There have been several unsuccessful attempts to reintroduce the species on Easter Island. The absence of a rhizobial inoculant strain for *S. toromiro*, has been highlighted as one of the causes of this species not being able to establish in Easter Island (Maunder et al., 2000; Jordan et al., 2001).

Sophora species have been reported to form mutualistic associations with soil bacteria, predominantly from the genus *Mesorhizobium* (De Meyer et al. 2015; De Meyer et al. 2016; Tan et al., 2015). Regarding the Chilean continental *Sophora* species, *Sophora cassioides* has been reported as able to form root nodules in association with rhizobia (Zuñiga-Feest et al., 2018) but there is no information on the diversity or identity of these root nodule bacteria and there is no history on nodulation on *S. macrocarpa*. This research work seeks to determine the specificity and nitrogen fixation effectiveness of the symbiotic associations of *Sophora toromiro* with different root nodule bacteria and to assess *Sophora toromiro* survival and performance on its natural environment

Root nodules were collected from *Sophora cassioides, S. macrocarpa and S. fernandeziana* from different areas of insular and continental Chile. The isolates were identified and assessed for nitrogen fixation effectiveness on *Sophora toromiro*, together with 10 strains from *Sophora* spp. from New Zealand. Plants were grown for 10 weeks in a phytotron at 22ºC and supplied weekly with N-free nutrient solution.

Diverse root nodule bacteria were isolated from *Sophora* spp. in Chile. *Sophora macrocarpa* root nodule bacteria were identified as *Mesorhizobium* spp., while *Sophora cassioides* was nodulated by *Bradyrhizobium* sp. The rhizobia from *Sophora fernandeziana* at Robinson Crusoe Island were identified as *Paraburkholderia* and *Mesorhizobium* spp. In the effectiveness experiment under controlled conditions, most of the Chilean *Sophora* isolates and all the strains from New Zealand *Sophora* induced nodulation on *Sophora toromiro*. The strain AG-275 from *Sophora macrocarpa* in Central Chile, and strains SMR3 and ICMP19512 from *Sophora microphylla* in New Zealand, were significantly different to other strains in terms of shoot dry weight and were categorized as effective in *Sophora toromiro.* Both *S. microphylla* and *S. macrocarpa* belong to the section Edwarsia and are phylogenetically closely related to *S. toromiro* (Peña et al., 2000).

At the Mataveri Otai nursery in Rapa Nui, an experiment was set up with toromiro plants inoculated with the outstanding strains from the phytotron experiment. Plant height, chlorophyll content and vigor were recorded. The inoculation with selected rhizobia allowed the survival of nearly 60% of the established plants in the last three years. Results in the field confirmed the compatibility between *S. microphylla* and *S. macrocarpa* rhizobia and *S. toromiro*. Plants inoculated with these effective strains are already 150 cm tall and some specimens have started to flower. The efforts on re-establishment of *S. toromiro* on its natural habitat come to collaborate to agreements signed by Chile such as the Convention on Biological Biodiversity (CBD). The achievement of this objective will mark an international precedent, in which a species classified by IUCN as extinct in the wild (EW) can be placed in a category of endangered.

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Novel root nodule symbionts of *Cicer arietinum* grown in the Ord River Scheme

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Key words

Nitrogen fixation, effectiveness, symbiotic gene transfer, genomic diversity

Abstract

Cicer arietinum (chickpea) is a recognized important commercial crop with Australia one of the top global exporters. In 2020-2021, 876.5 kt of chickpea was produced in Australia, with 7.4 kt in Western Australia (ABS, 2022). In Australia, chickpea has been inoculated since the 1970's with *Mesorhizobium ciceri* sv. *ciceri* CC1192 or Group N, a highly effective strain from Israel (Corbin et al., 1977). CC1192 has been shown to harbor a mobile 419-kb symbiosis integrative conjugative element or ICE (ICE*Mc*Sym[1192]) which can lead to diverse unrelated *Mesorhizobium* becoming symbiotic with chickpea (Hill et al., 2020). In this work, 123 authenticated chickpea strains were obtained from nodules of plants growing in 11 different irrigated paddocks within the Ord River scheme agricultural region in 2017 (Figure 1).

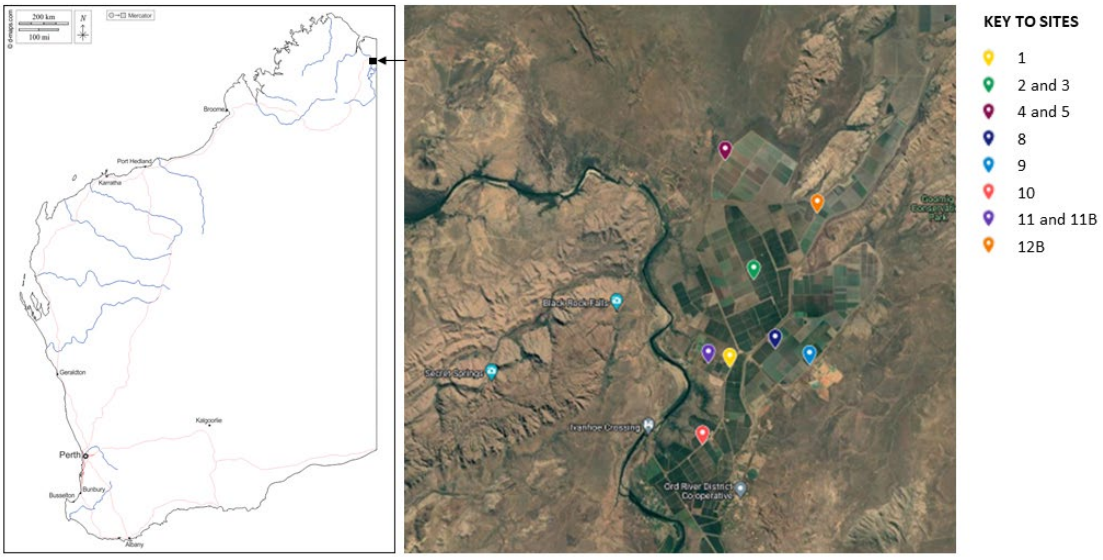

Figure 1. Ord River scheme agricultural region sites at which chickpea were collected and sampled for nodules.

All the 123 strains were shown to carry the ICE*Mc*Sym[1192] and of these, 97 strains were shown to be genetically distinct from CC1192 using the RPO1-PCR primers (Richardson et al., 1995). The strains were typed into 12 different fingerprint groupings. A representative strain of each group was selected and when assessed for effectiveness on chickpea with CC1192 only one strain was found to be significantly poorly effective (Figure 2). Two strains, K11a2 and K102b1, which between them represent 41.2% of the total novel strains, were identical in dry top weights produced. This survey demonstrates that ICE transfer does not necessarily yield ineffective microsymbionts as previously

observed with *Mesorhizobium* inoculants (Nandasena et al., 2007). Genomic sequencing on selected type strains shows that they are diverse and distinct from the *M. ciceri* clade and strains described by Hill et al. (2020).

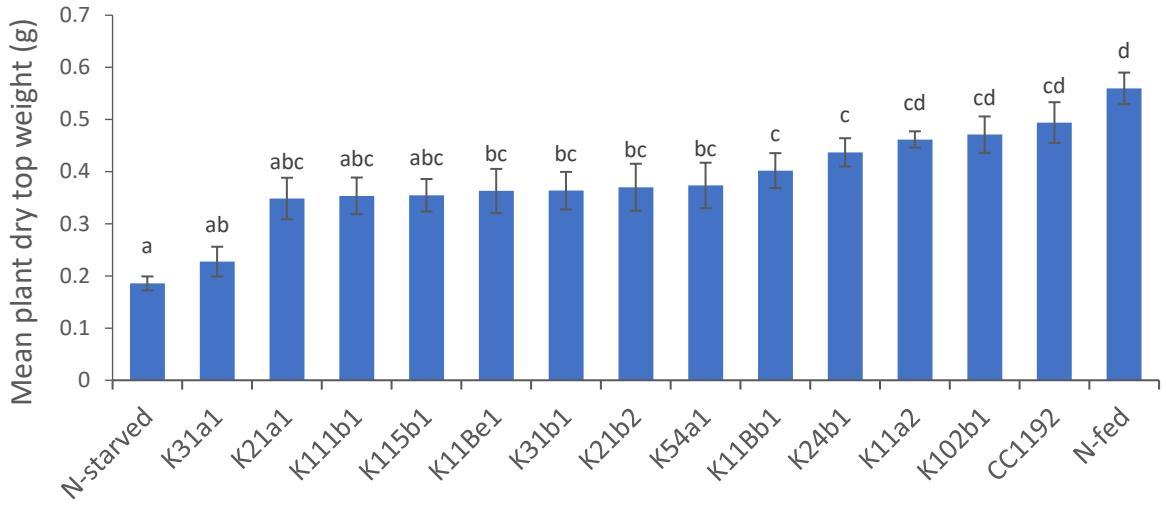

Figure 2. Mean plant dry top weight of *Cicer arietinum* inoculated with novel strains from the Ord River scheme region. Treatments are shown with standard error of means and those treaments that share a letter are not significantly different according to the Tukey HSD test ($P \leq 0.05$).

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Diversity and field symbiotic performance of *Mesorhizobium* strains collected across chickpea cropping areas of Australia and Myanmar

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Abstract

There has been increasing interest in the diversity of chickpea-nodulating rhizobia throughout the major chickpea producing countries around the world. In particular, the role of inoculation and presence of diverse *Mesorhizobium* species in the soil that form effective symbiosis with chickpeas have been studied. Newly isolated *Mesorhizobium* strains collected from across Australian and Myanmar cropping regions were analysed in a pot experiment to evaluate nodulation and symbiotic effectiveness (SE%) in chickpea plants. Phylogenetic analyses revealed chickpea nodulating rhizobia in Myanmar soils were most closely related to *M. gobiense, M. muleiense, M. silamurunense, M. tamadayense* and *M. temperatum*. Around two-thirds of the Myanmar strains (68%) were most closely related to Indian strain IC-2058 (CA-181), which is also most closely related to *M. gobiense*. There were no strains that were closely related to the cognate rhizobial species to nodulate chickpea: *M. ciceri* and *M. mediterraneum*. This is in contrast to the Australian soils, which were dominated by *M. ciceri*, *M. temperatum* and *M. huakuii*. The only co-occurring species found in both Myanmar and Australia were *M. tamadayense* and *M. silumurunense*. Continued inoculation with CC1192, which originated in Israel, may have reduced diversity of chickpea strains in Australian soils.

Strains from the above isolations were selected from symbiotic effectiveness studies conducted in the glasshouse and were tested in field trials in 4 southern Australian environments over 2 years. It was found that the strains isolated from Myanmar soils were inferior in nodulation compared to the Australian strains and CC1192. Strains collected from Australian soils had superior in survival on seed, improved nodulation and shoot dry weight at most experimental sites, compared with strains isolated from Myanmar soils. At most field sites, the newly isolated strains did not perform better than CC1192. Strain A47, collected from Australia, was the most effective of all the strains tested in this study, with improved symbiotic N_2 fixation at the Angas Valley site. Australian strain A78 showed equally good nodulation with CC1192 on a highly acidic soil (pH 4.18 $CaCl_2$) at the Kapunda site, while all other test strains had inadequate nodulation at that site. Although Myanmar strains performed poorly in Australian environments, they have not been tested in Myanmar environments, where high pH vertisol soils and high temperatures are common and should be evaluated there in future.

Complete genome sequencing and phylogenetic analysis of the Australian commercial rhizobial inoculants

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Key words
Inoculant, sequencing, genomics, horizontal gene transfer

Abstract
Rhizobia compatible with crop and pasture legumes aren't naturally present in Australian soils, which has led to the introduction of many highly effective nitrogen-fixing bacteria from other parts of the world as commercial inoculant strains. Rhizobial symbiosis genes (*nod*/*nif*/*fix*) are encoded from mobile genetic elements, such as plasmids or integrative and conjugative elements (ICEs). As a result they are highly mobile and able to transfer between strains.

Recently, it has become increasingly clear that the genetic diversity of strains nodulating legumes far exceeds the diversity of strains introduced as inoculants (Demezas, Reardon et al. 1995; Hebb, Richardson et al. 1998; Ballard, Charman et al. 2004; Stepkowski, Moulin et al. 2005). This could partly be explained by horizontal transfer of symbiosis genes between inoculants and pre-existing soil bacteria, creating novel hybrid rhizobia (Nandasena, O'Hara et al. 2007; Hill, Colombi et al. 2021). It could also be due to the accumulation of mutations giving rise to newly evolved rhizobia.

Given this genetic instability, a blueprint for inoculant genomes is important to maintain the integrity of this crucial national resource. However, we lack the genomic data on the commercial legume inoculants needed to track changes in genome structure and content as well as the technology to easily identify strains within nodules. In this project, we describe the complete genome sequencing of the Australian commercial rhizobial inoculants, with special attention paid to the symbiosis genes, and examine their relationship to each other, as well as to the broader set of publicly available genome sequences. This critical baseline data can be used to address fundamental issues surrounding inoculant usage including the identity of the bacterium within a nodule and how the inoculants change over time.

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SESSION 8 – EVOLUTION AND DIVERSITY OF RHIZOBIA II

Chair: Professor Philip Poole, University of Oxford

Diverse populations of nonsymbiotic *Mesorhizobium* spp. present in soils have a capacity to become legume symbionts following horizontal gene transfer

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Key words

Mesorhizobium, nitrogen fixation, symbiosis, plant-microbe interactions, horizontal gene transfer, ICE, integrative and conjugative elements, conjugation, soil bacteria, rhizosphere, symbiosis island, evolution

Abstract

Mesorhizobia are soil bacteria that establish nitrogen-fixing symbioses with various legumes. Novel symbiotic mesorhizobia frequently evolve following horizontal transfer of symbiosis-gene carrying integrative and conjugative elements (ICESyms) to indigenous mesorhizobia in soils. Evolved symbionts exhibit a wide range in symbiotic effectiveness, with some fixing nitrogen poorly or not at all (Sullivan et al. 1995; Nandasena et al. 2006; Hill et al. 2020). Little is known about the genetic diversity and symbiotic potential of indigenous soil mesorhizobia prior to ICESym acquisition. Here we sequenced genomes of 144 *Mesorhizobium* spp. strains cultured directly from cultivated and uncultivated Australian soils. Of these, 126 lacked symbiosis genes. Isolated symbiotic strains were either exotic strains used previously as legume inoculants, or indigenous mesorhizobia that had acquired exotic ICESyms. No native symbiotic strains were identified. Indigenous nonsymbiotic strains formed 22 genospecies with phylogenomic diversity overlapping the diversity of internationally isolated symbiotic *Mesorhizobium* spp.. The genomes of indigenous mesorhizobia exhibited no evidence of prior involvement in nitrogen-fixing symbiosis, yet their core genomes were similar to symbiotic strains and they generally lacked genes for synthesis of biotin, nicotinate and thiamine. Genomes of nonsymbiotic mesorhizobia harboured similar mobile elements to those of symbiotic mesorhizobia, including ICESym-like elements carrying aforementioned vitamin-synthesis genes but lacking symbiosis genes. Diverse indigenous isolates receiving ICESyms through horizontal gene transfer formed effective symbioses with *Lotus* and *Biserrula* legumes, indicating most nonsymbiotic mesorhizobia have an innate capacity for nitrogen-fixing symbiosis following ICESym acquisition. Non-fixing ICESym-harbouring strains were isolated sporadically within species alongside effective symbionts, indicating chromosomal lineage does not predict symbiotic potential. Our observations

suggest previously observed genomic diversity amongst symbiotic *Mesorhizobium* spp. represents a fraction of the extant diversity of nonsymbiotic strains. The overlapping phylogeny of symbiotic and nonsymbiotic clades suggests major clades of *Mesorhizobium* diverged prior to introduction of symbiosis genes and therefore chromosomal genes involved in symbiosis have evolved largely independent of nitrogen-fixing symbiosis.

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Engineering nitrogen fixing symbiosis between cereals and bacteria

Timothy Haskett[1], Ponraj Paramasivan[2], Barney Geddes[1,3], Marta Mendes[1], Amelie Joffrin[4], Amber Thompson[4], Kirsten Christensen[4], Patrick Green[1], Beatriz Jorrin[1], Hayley Knights[1], Min-Hyung Ryu[5], Ramakrishnan Karunakaran[6], Marcelo Bueno Batista[6], Paul Brett[7], Ray Dixon[6], Christopher Voigt[5], Giles Oldroyd[2,8] and Philip Poole[1]

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Key words
Nitrogen fixation, ammonia excretion, plant-microbe interactions, synthetic biology, rhizopine

Abstract
Inoculation of cereals with diazotrophic bacteria could provide a sustainable alternative to the application of nitrogen (N) fertilizers in agriculture. However, due to the costly energy demands of N fixation, diazotrophic bacteria have evolved complex regulatory networks that permit expression of the catalyst nitrogenase only under conditions of N starvation, whereas the same condition stimulates upregulation of high-affinity ammonia (NH_3) assimilation by glutamine synthetase (GS), preventing excess release of excess NH_3 for plants. Diazotrophs can be engineered to excrete NH_3 by interference with GS, however control is required to minimise fitness penalties and prevent unintended provision of NH_3 to non-target plants (Haskett et al., 2020). We have engineered novel *trans*-kingdom signalling between plants and bacteria which, through plant production of the bacterial signal rhizopine, permits control of bacterial gene expression in association with the plant (Geddes et al., 2019). We used rhizopine signalling to establish control of the nitrogenase master regulator *nifA* and the N metabolism sigma-factor *rpoN* in our model strain *Azorhizobium caulinodans*, which drove nitrogenase expression and activity by bacteria colonizing rhizopine producing (*RhiP*) barley roots (Ryu et al., 2020; Haskett et al., 2022b). Because the strain did not release NH_3 derived from N-fixation, we additionally engineered into the strain a unidirectional adenylyl transferase under NifA control that drove shutdown of GS and release of NH_3 when rhizopine was added in culture (Haskett et al., 2022a). GS inactivation prevented the production of glutamine from NH_3, which completely alleviated negative feedback regulation on nitrogenase. This barley-*Azorhizobium* model system now represents a prototype "synthetic plant-controlled symbiosis" in which the bacteria engage N fixation and NH_3 excretion specifically when sensing rhizopine produced by target plants.

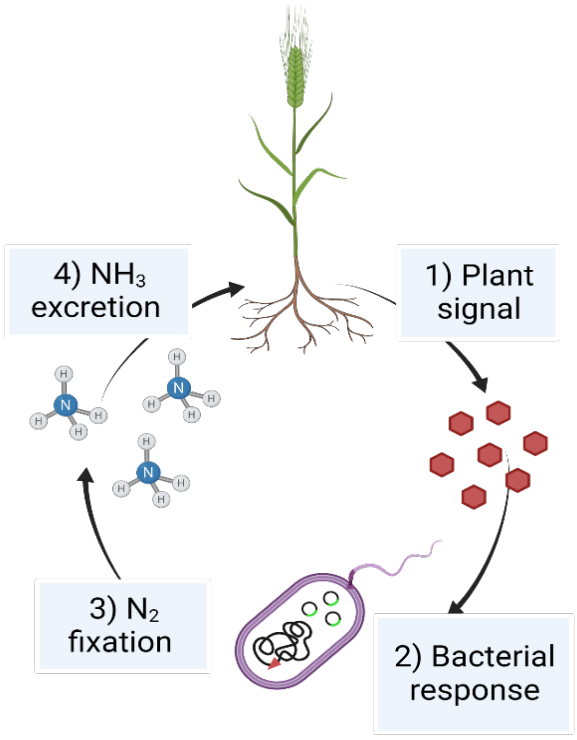

Figure 1. Synthetic nitrogen-fixing symbiosis between *Azorhizobium caulinodans* and rhizopine producing (*RhiP*) barley

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Progress towards engineering nitrogenase directly into crops

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Key words
Metalloenzymes, synthetic biology, mitochondria, chloroplasts

Abstract
Modern cropping is highly dependent upon addition of industrial N fertiliser, with half of the protein in our bodies derived from Haber Bosch N. Public interest in sustainable cropping combined with recent increases in the costs of fertiliser have rekindled interest to finding more sustainable approaches to feeding the global population. Since the early 1970's an ambition of plant biotechnologists has been engineering non-legumes with the ability to produce their own N fertilizer. Some of these approaches can be broadly categorised as engineering nodulation into non-legumes; engineering commensal bacteria to fix N_2 within plant tissues; and directly embedding the entire pathway for nitrogenase into the genome of crops. For this last option here we provide an overview and update for recent progress and future challenges for engineering nitrogenase directly into crops.

Nitrogenase is unique in nature and its biochemical and genetic features need to be considered by synthetic biologists. Key challenges include protection of the enzyme from oxygen, provision of electrons and ATP, supply of metals for co-factor synthesis and the sheer number of genes and protein components required for this metalloenzyme to be assembled and for catalysis. After 50 years of research there have been some remarkable breakthroughs in the last five years. A key theme is the sub-cellular re-location of the enzyme to mitochondria or chloroplasts, where the majority of endogenous metalloenzymes are made in plants. From these locations synthetic biologists have demonstrated activity for nitrogenase components including FeProtein (NifHMSU) and co-factor synthesis (NifB, FdxN and NifV) and stability of the reductase (NifDK). These breakthroughs used differing approaches including rational enzyme design, naturally occurring variants and combinatorial gene expression. The most recent literature (see below)and results from our laboratory will be presented. An outline of areas where insights from the naturally evolved diazotrophic symbioses could be applied to successfully engineer nitrogenase into crops will also be discussed.

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A superhelical-filament-forming DNA-binding protein controls horizontal transfer of symbiosis genes

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Keywords

RdfS, excisionase, Integrative and Conjugative Elements, Mesorhizobia

Abstract

Integrative and conjugative elements (ICE) are mobile genetic elements that can excise from bacterial chromosomes and horizontally transfer via conjugation. Symbiosis ICEs from *Mesorhizobium* spp. carry genes that convert recipients into nitrogen-fixing legume symbionts. RdfS is a critical DNA-binding protein which controls the chromosomal excision and horizontal gene transfer of symbiosis ICEs. Here we present the X-ray crystal structure of RdfS and reveal it forms superhelical filaments in head-to-tail orientation and a continuous positively charged surface likely contacting DNA. RdfS contains a winged helix-turn-helix (wHTH) DNA-binding domain with a highly disordered C-terminus. Uniquely, RdfS is the first structurally characterised wHTH carrying an additional N-terminal helix which contributes to the oligomerisation interface within helical filaments in crystals. Using DNA-binding assays we demonstrated RdfS binds the DNA recombination region *attP* and promoter regions to control gene transfer. DNA-shift patterns were consistent with concentration-dependent oligomerization of RdfS in complex with DNA. While RdfS bound several individual sites with high specificity, no clear binding consensus sequence was identified, suggesting oligomerization state and DNA topology may augment RdfS affinity to specific DNA sites. The discovered structural oligomerization and mode of RdfS DNA-binding are consistent with the multifunctional and concentration-dependent control RdfS has over ICE excision, conjugation, transcriptional regulation and growth inhibition.

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Bacterial cell-cell signaling regulates a network of non-coding RNAs in *Mesorhizobium japonicum* and *M. ciceri*

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Keywords

Quorum sensing, crotonase, small non-coding RNA

Abstract

Mesorhizobium japonicum is a nitrogen fixing symbiont of *Lotus sp*. (Sullivan and Ronson, 1998) and has been extensively used in the studies of pant-microbe interactions including the genetic mechanisms of symbiotic nitrogen fixation and horizontal DNA transfer of symbiosis genes.

Bacteria use intercellular chemical signaling systems called quorum sensing (QS) to control various phenotypes including those involved in plant-symbiosis and horizontal gene transfer. For LuxRI-Family QS systems, cells produce membrane-diffusible molecules called *N*-acyl-homoserine lactones (AHLs) which increase in concentration with cell density. AHLs bind cytoplasmic receptor proteins in neighboring cells, which then activate or derepress phenotypes under QS control (Waters and Bassler, 2005). The TraR/TraI QS system has been characterized in *M. japonicum* R7A and controls transfer genes responsible for nitrogen-fixation and symbiosis (Ramsay et al., 2009). We have identified a separate, novel QS locus conserved across the genus *Mesorhizobium*, named the *Mesorhizobium* quorum-sensing locus (MQS).

Distinct from all LuxRI-family QS systems, MQS encodes a second AHL synthase gene *mqsC* in addition to *mqsI*, both of which are required for activation of the MQS receptor MqsR. This second AHL synthase protein – MqsC – is related to the crotonase family of enzymes, which in other bacteria produce a distinct family of unsaturated fatty-acid QS molecules called diffusible signal factors (DSF) (Zhou et al., 2016). This unique MQS system appears to represent an evolutionary amalgamation of these two enzymes for production of AHL molecules containing unsaturated fatty acids.

Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was used to detect AHL molecules produced by the MQS locus of *M. japonicum* R7A, which identified the long-chain unsaturated AHL molecule – 5-*cis*-C12-HSL. Chemically synthesized 5-*cis*-C12-HSL activated the MqsR system in the absence of *mqsI* and *mqsC*.

We were unable to identify roles for the MQS system in gene transfer or symbiosis, however, RNA sequencing of *M. japonicum* R7A and *M. ciceri* CC1192 identified 6 MQS-regulated non-coding RNAs (sRNAs). Each of the sRNA promoter regions contained a conserved inverted repeat sequence; likely the binding site for MqsR. Promoter-*lacZ* fusions confirmed expression of each sRNA was dependent on MqsR and 5-*cis*-C12-HSL. Work is underway to determine the physiological role of this sRNA network. The genomic locations of sRNA genes and the *mqsRIC* genes suggests they may be associated with phosphate uptake and/or metabolism.

In summary, we have identified a QS system conserved throughout the genus *Mesorhizobium* that produces an unsaturated 5-*cis*-C12-HSL molecule. The system uniquely requires two synthesis genes and regulates several small non-coding RNAs, so far of unknown function.

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SESSION 9 – INOCULATION TECHNOLOGIES AND PRACTICE I

Chair: Dr David Herridge, University of New England

Providing a secure facility to store rhizobia germplasm of global value

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Key Words
Rhizobia, inoculants, Genebank

Abstract
N_2 fixed by pulse, oilseed, pasture and forage legumes is valued at approximately $12 billion pa globally, with much of this N_2 fixed by rhizobia supplied as inoculants. The success of legume inoculation is underpinned by the availability of high-quality strains of rhizobia. These strains are the result of countless hours of work involving collection, isolation, authentication and evaluation. International gene-bank collections of rhizobia strains are therefore a priceless resource. Over the last ~70 years of rhizobia research, many rhizobia collections have been created, and these have tended to have regional or national focus, targeting genera or species of rhizobia to service legumes grown in that region.

In Australia, where all agricultural legumes are exotic, these collections have been pivotal to the profitability and adaptability of farming systems, with numerous exotic strains sourced internationally from edaphically matched locations. Recent geopolitical instability in many source countries and an increase in international restrictions on the collection and importation of biological materials, further underline the critical nature of these collections. However, when institutions change research priorities, research funding ceases or curators retire without an effective transition plan, these valuable collections are put in jeopardy.

To provide a repository for these valuable collections of rhizobia, the International Legume Inoculant Genebank (ILIG) has been established under the custodianship of Legume Rhizobium Sciences (LRS) at Murdoch University and funded by the Australian Grains Development and Research Corporation (GRDC). The purpose-built facility (Figure 1) housing the ILIG collection is based at Murdoch University, Perth in Western Australia, and contains lyophilised strains sourced from local, national and global locations. With a capacity to hold 31,000 strains, the ILIG currently contains more than 11,500 strains representing at least 103 species of rhizobia, isolated from 708 legume species, collected from 92 countries around the world.

The ILIG collection has been formed from a consolidation of strains from 33 national and international sources including the Western Soil Microbiology (WSM), CB, CC, WU, ARR, RAD, USDA, NifTAL, ICARDA, CIAT and ICRISAT collections. The infrastructure for storage of these strains is now in place, and the

public online catalogue is live, and being populated with all available metadata (e.g. strain name, year, place and host of isolation, phenotypic data, where available). Genome sequencing of all 43 Australian commercial inoculant strains is nearing completion, as is a site for the physical backup for this collection (Northam DPIRD facility, Western Australia).

Figure 1. Photographs of the ILIG facility at Murdoch University. The facility has the capacity to hold 31,104 strains in glass ampoules, with comparable -80°C storage.

With the establishment phase of the ILIG now well matured, there is a need to integrate any strains available from other national and international collections into the ILIG and connect with curators of other rhizobia collections with a view to forming a rhizobium strain collection network. Moving forward, this working collection housing the largest and most diverse set of rhizobia strains in the world will be a critical source of new inoculant strains as well as providing a resource for researchers studying legume-rhizobia symbioses. Ensuring this newly constructed and developed collection is adequately supported in the future is of paramount importance. An overview of the ILIG will be presented and some of the progress towards tackling these challenges will be discussed.

Persistence of soybean *Bradyrhizobium* inoculants in the soil and impacts on soil microbial diversity as determined by soil nodulation trapping and shotgun metagenomics studies

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Key words

Bradyrhizobium, nodulation, soil persistence, soybean cultivar, metagenomics

Introduction

Due to the absence of the specific *Bradyrhizobium* species that nodulates soybean in South Africa, a few exotic strains were introduced in the late 1960s, with *Bradyrhizobium japonicum* strain WB74 (= SARCC-340 = CB1809^T) the latest to be released in 1998. Since the 1960s however, there has been a sharp increase in the number of soybean cultivars in the country. Moreover, several imported inoculant products have since been introduced without being tested under South African soil conditions. This necessitated the selection of more efficient rhizobia through cultivar-strain nodulation compatibility and nitrogen fixation efficacy study across a wide range of commonly used soybean cultivars. Here we present the first part of the study, persistence of *Bradyrhizobium* strains from past inoculation, their ability to colonize and nodulate different soybean cultivars in a soil trap experiment and their impact on native microbial diversity using metagenomics approach.

Results and Discussion

The persistence of *Bradyrhizobium* populations in the soil eight years since the last inoculation and the impacts of the established *Bradyrhizobium* population on native microbial diversity was investigated. When soybean cultivars were planted in soils collected from the different sites to investigate nodulation and the identity of the trapped rhizobia, many of the cultivars formed very conspicuous and pink nodules mainly on the crown region (Hassen et al. 2022). Using sequence analysis of the 16S rRNA of the extracted DNA of the nodule isolates, we found out that ≥ 80% of the isolates belonged to *Bradyrhizobium japonicum* indicating a huge establishment of *B. japonicum* population in those soils not inoculated with any commercial rhizobia inoculants since 2014. A comparative metagenomics approach was used to investigate if this *Bradyrhizobium* establishment has brought any effect on the native soil microbial diversity using soils from the soybean farm and the soil from the maize plot which never received any rhizobium inoculation for several years. Taxonomic distribution analysis of the soil from the soybean farms using contig LCA algorithm on MG-RAST (Meyer et al. 2008) shows the genus *Bradyrhizobium* has the highest abundance (9%) in the entire soil microbial community. The analysis for the maize rhizosphere soil however indicates that the genus *Bradyrhizobium* is only 2.03% compared to other genera such as *Rubrobacter, Nitrospora*, *Streptomyces, Mycobacteria* and others (Figures 1A and B). The data generated here provides valuable information both on the establishment of soybean *Bradyrhizobium* strains in native soils and their impact on indigenous microbial population. The study is also of considerable importance in the quest

for selection and development of effective soybean rhizobia inoculants for use on different soybean cultivars in South Africa.

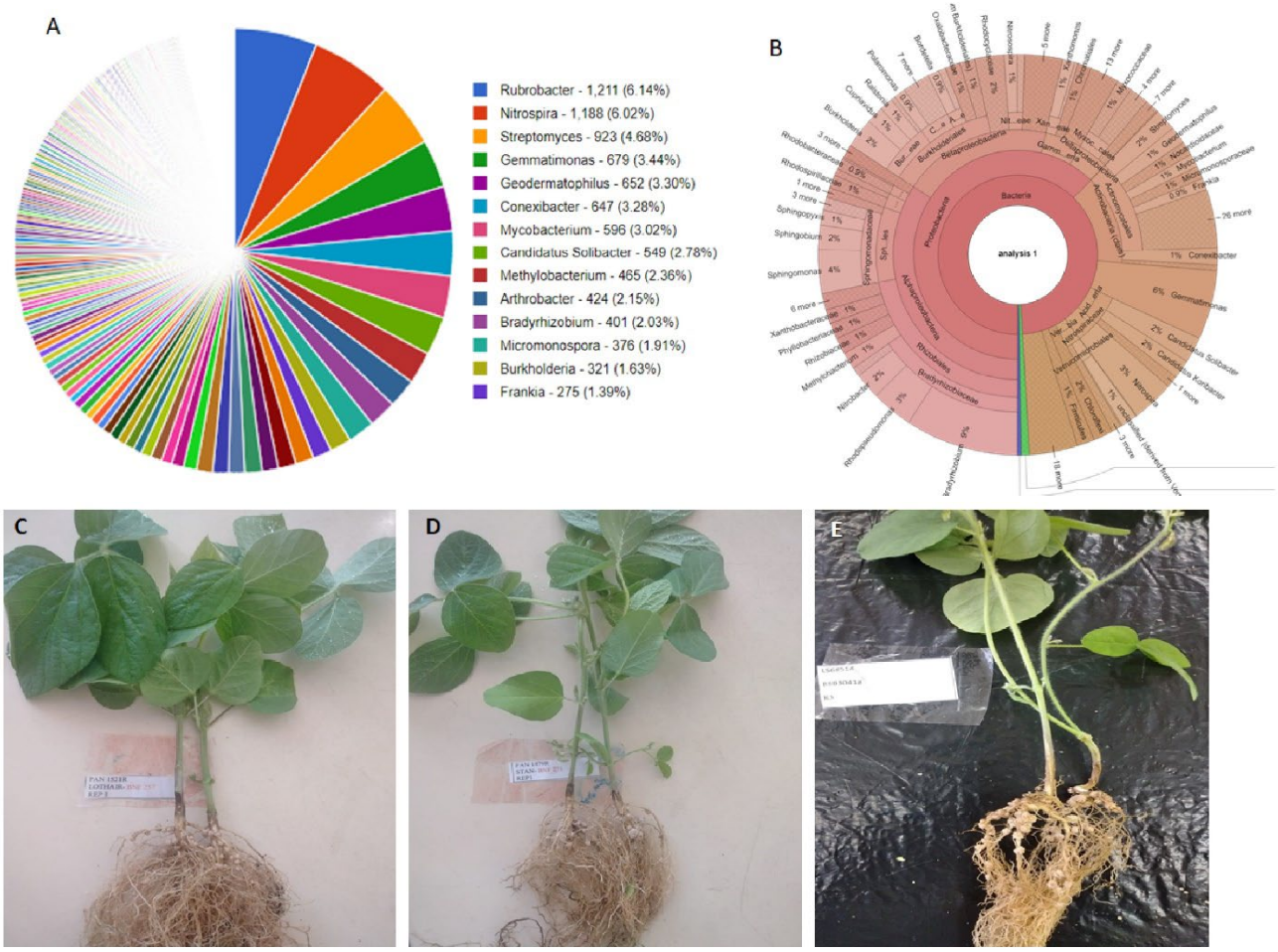

Figure 1. Taxonomic abundance to the genus level of the soil microbial diversity of maize rhizosphere soil not inoculated at any time (**A**). Krona chart showing the genus level taxonomic abundance of soybean field inoculated with rhizobia until 2014 only (**B**). Several pink and big nodules formed in by different soybean cultivars after seeds were planted in soils collected from soybean farm rhizosphere in a glasshouse soil trap pot experiment (**C, D, and E**).

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Cross-compatibility and genetic stability of rhizobia to maximise nitrogen fixation in the new annual pasture legume *Scorpiurus muricatus*

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Key words

Inoculant development, *Mesorhizobium*, *Scorpiurus muricatus*

Abstract

Legumes play an integral role in increasing agricultural productivity, especially in low input agricultural regions of Australia, due to their symbiotic interactions with the soil bacteria called rhizobia. In the medium-to-low rainfall areas of southern Australia there is a lack of suitable annual pasture legumes, which is limiting agricultural productivity and profitability. *Scorpiurus muricatus* is an annual pasture legume classified in tribe *Loteae* from the Mediterranean region with a broad tolerance of soil type, pH and nutrient status, and a high drought tolerance, potentially making it a suitable pasture legume for medium-to-low rainfall areas of southern Australia. Crucial to the introduction of *S. muricatus* is the identification of a highly effective rhizobial inoculant strain, which does not ineffectively nodulate non-target legumes. At present, there is very little information available on the diversity, effectiveness and host-range of rhizobia that form a symbiosis with *S. muricatus*.

A total of 50 strains have been isolated from soils, with a history of *S. muricatus*, from Australia (5), Croatia (3), Israel (12), Morocco (4) and Sardinia (26). Initial genotyping of these strains using random amplified polymorphic DNA fingerprinting with the RPO1 primer showed at least 39 unique strains. Further core genome analysis following Illumina sequencing identified 36 *Mesorhizobium* strains which grouped with type strains isolated from Argentina: *M. sanjuanii*^{T}; Australia: *M. opportunistum*^{T}; China: *M. muleinse*^{T} and *M. tianshanense*^{T}; and the Mediterranean region: *M. ciceri sv biserrula*^{T}, *M. delmotii*^{T}, *M. mediterraneum*^{T}, and *M. prunaredense*^{T}. These data show the high level of diversity of *S. muricatus* nodulating *Mesorhizobium*.

To investigate the relatedness of the core nodulation genes of these strains to each other and to *Biserrula pelecinus, Cicer arietinum* and *Lotus* spp. nodulating strains, a phylogenetic tree was constructed using *nodABCIJ*. The resulting phylogenetic tree grouped 34 of 36 strains with Lotus nodulating (*M. loti* DSM2626^T, *M. japonicum* MAFF303099^T and *M. japonicum* R7A) and broad host-range *M. ciceri* WSM1293 and *M. ciceri sv biserrulae* WSM1284) strains. In contrast, no strains grouped with the narrow host range *Cicer arietinum* (*M. ciceri* CC1192, *M. ciceri* CMG6) and *Biserrula pelecinus* (*M. ciceri sv biserrulae* WSM1271^T and *M. ciceri sv biserrulae* WSM1497) nodulating strains. These groupings suggest that *S. muricatus* strains may have a broad host-range, and this is being investigated through nodulation assays of a subset of six strains with eight legumes (*B. pelecinus*, *Bituminaria bituminosa*, *C. arietinum*, *Hedysarum coronarium*, *Lotus corniculatus*, *Lotus ornithopodioides*, *Lupinus angustifolius* and *Ornithopus sativus*) grown in Australian farming systems.

S. muricatus promiscuity has been investigated by testing whether *Mesorhizobium* and *Bradyrhizobium* inoculants nodulate this legume. *L. corniculatus* and *L. ornithopodioides* inoculants, SU343 and WSM1293 respectively, nodulated and fixed N_2 on *S. muricatus*. In contrast, *B. bituminosa, B. pelecinus* and *C. arietinum* inoculants WSM4083, WSM1497 and CC1192 respectively, did not nodulate *S. muricatus*. *Bradyrhizobium* inoculants for *Lotus pedunculatus* (CC829), and *Lupinus* spp. and *Ornithopus* spp. (WSM471 and WU425) formed few, ineffective pink/green nodules. The *Bradyrhizobium* inoculants CB756 and CB1809 (*Glycine max*) did not nodulate *S. muricatus*. These experiments have shown *S. muricatus* is a promiscuous host and may be impacted in agricultural systems where legumes have been grown and inoculated previously.

To date the 23 *S. muricatus* nodulating *Mesorhizobium* strains tested fix N_2 on *S. muricatus*, producing equivalent shoot dry weight/plant to the reference strain *M. opportunistum* WSM1386 which is 60% of the N-fed control. Further research is needed to identify a suitable inoculant strain for the introduction of *S. muricatus* as a new pasture legume for the medium-to-low rainfall areas of southern Australia.

SESSION 10 – INOCULATION TECHNOLOGIES AND PRACTICE II

Chair: Professor John Howieson, Murdoch University

The replacement of WSM1455 (Group E/F) with WSM4643 for on farm inoculation of *Pisum sativum*, *Lens culinaris* and vetch (*Vicia sativa* and *V. villosa*)

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Key words
Legumes, rhizobia, nitrogen fixation, inoculants, acidic soils

Introduction

The micro-symbiont *Rhizobium leguminosarum* biovar *viciae* forms symbiosis with many grain legumes including *Pisum sativum* (field pea), *Lens culinaris* (lentil) and *Vicia faba* (faba bean), as well as the forage species of *Vicia* spp. (e.g. vetch) and *Lathyrus* spp. (e.g. grass pea). However, whereas *P. sativum* and forage *Vicia* spp. consistently form highly effective symbioses with a subset of elite strains of this biovar, *L. culinaris* and *V. faba* often show sub-optimal symbioses (Herridge, 2008; Howieson, 1999). This was reflected in the separation of the commercial *R. I. viciae* inoculants in Australian agriculture (Herridge, 2008). Until recently *P. sativum*, the forage *Vicia* spp. and *Lathyrus* spp. were inoculated with strain SU303 (Group E), whereas the pulse legumes *L. culinaris* and *V. faba* were inoculated with strain WSM1455 (Group F; Farquharson et al., 2022). However, SU303 which is recommended for *P. sativum* and *Vicia* spp. (vetch), is difficult to manufacture and is not regularly available or used by industry. Consequently, this group of legumes are inoculated with WSM1455 (commercial strain Group F). Importantly, there was evidence that legumes inoculated with SU303 performed poorly in infertile, acidic soils in southern Australia (Evans, 2005). In addition, evaluation was necessary with the use of WSM1455 inoculated on *P. sativum* and *Vicia* spp. (vetch) in these soils. For this reason, a research project was initiated to identify new strains better suited to these soils, on which producers are hoping to expand pulse sowings.

Additional strain germplasm was required because of a limited number of strains fitting the basic criteria (broad host range, full effectiveness) in the International Legume Inoculant Genebank (ILIG) managed by Legume Rhizobium Sciences, Murdoch University. This deficiency was addressed by collecting nodules from *P. sativum* plants grown in low pH soils from southern Italy, a recognised centre of origin (Yates et al., 2021a). As a result of a thorough evaluation, a new elite strain with

superior symbiotic performance and saprophytic competence, WSM4643, emerged across multiple experiments (Yates et al., 2021b).

Results

WSM4643 has proven to grow well in commercial culture, exceeding minimum numerical standards at manufacture and after storage. The strain displayed no loss of symbiotic capacity over serial sub-culture *in vitro* and thus is genetically stable. Strain WSM4643 and WSM1455 were equivalent for N-fixation capacity and broad host range in 8 glasshouse experiments, effectively nodulating *P. sativum*, *L. culinaris*, *Vicia* spp. (vetch) and *V. faba*. Further, WSM4643 was shown to be superior to WSM1455 in persisting and colonising target soils (saprophytic competence). Additionally, in 47 field trials conducted throughout WA and NSW on predominantly acidic soils (pH_{Ca}<4.9) between 2015 and 2021 strain WSM4643 outperformed WSM1455 as an inoculant for *P. sativum*, *L. culinaris* and *Vicia* spp. (vetch) in 41% of parameters quantifying nodulation, early biomass, peak biomass, N fixation, grain yield and grain protein.

Discussion

It is recommended that WSM4643 replace WSM1455 and SU303 as the national inoculant for *P. sativum*, *L. culinaris* and *Vicia* spp. (vetch) in the Group E packaging. The above recommendation is made in conjunction with the National Rhizobium Steering Committee (NRSC) endorsed change to Group F in which strain SRDI969 will be nationally recommended as the inoculant for tick and broad bean (*Vicia faba*). Field trials in 2022 highlighted that WSM4643 is highly effective with *Vicia faba* but this requires a final analysis of measurements.

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Assuring high quality of legume inoculants: maximising the potential of legume inoculants for Nitrogen fixation from the start

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Key words

Inoculant quality, commercial rhizobia, nitrogen fixation, legumes

Abstract

The Australian Inoculants Research Group (AIRG) within the New South Wales Department of Primary Industries (NSW DPI) independently tests legume inoculants against the standards outlined in the National Code of Practice (CoP) for legume inoculant quality, with passing batches awarded a 'Green Tick'. Products that carry AIRG's quality assurance Green Tick logo have undergone independent testing to ensure they contain the correct strain and minimum number of viable root-nodule micro-organisms (rhizobia) as labelled at the point of manufacture and can nodulate a host legume. This paper presents the results of independent inoculant testing of 428 batches of legume inoculants between 2019-2022. Most batches tested were peat-based legume inoculants. Granular, liquid and freeze-dried product was also tested.

In 2019, the Australian Inoculants Research Group commenced routinely testing granular product under the 'Green Tick' quality assurance program. From July 2019 to March 2022, peat inoculants achieved a 'pass' grade on average 92.04% of the time. Of the small percentage of batches that failed to reach quality standards, 4.87% failed due to contamination i.e. detection of non-rhizobial organisms at manufacture. Only 3.10% of the tested peat batches failed due to low counts i.e. less than 1×10^9 CFU/g peat at manufacture as per the National CoP.

In summary, peat-based inoculants in the Australian marketplace are of excellent quality at manufacture. Producers can be confident that a product purchased in store endorsed with a 'Green Tick' is optimised for success because it has been quality assured via the rigorous, independent testing process of the Australian Inoculants Research Group (AIRG).

Use of DNA tests to quantify the number of *Rhizobium leguminosarum* bv. *viciae* and *Mesorhizobium ciceri* in field soils of varying pH

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Key words
Pea, chickpea, MPN, soil acidity, qPCR

Introduction
The number of rhizobia present in soil is a key determinant of legume nodulation, growth and nitrogen fixation. Where numbers are low or absent, rhizobia must be delivered as inoculants applied to the seed or soil.

Low soil pH is recognised as detrimental to the survival of acid sensitive rhizobia. With soils continuing to acidify and pulse crops increasingly being grown in marginal environments, understanding the requirement for inoculation on acidic soils will help growers to minimise rhizobia constraints to pulse production.

The development of quantitative DNA tests (qPCR tests) to estimate rhizobia number in soil has enabled large scale surveys to be conducted and provides the basis for a service to growers to determine inoculation requirements. qPCR tests are now available for *Rhizobium leguminosarum* bv. *viciae* (nodulating field pea, faba bean, lentil and vetch) and *Mesorhizobium ciceri* (nodulating chickpea). Here, we provide an example where the qPCR tests have been used to investigate the relationships between soil pH and number of *Rhizobium leguminosarum* bv. *viciae* and *Mesorhizobium ciceri* in soils.

Methods
qPCR assays (TaqMan MGB) specific for *Rhizobium leguminosarum* bv. *viciae* or *Mesorhizobium ciceri*, hereafter referred to as *Rlv* or *Mc*, respectively, were used to estimate the number of *Rlv* and *Mc* in 1091 stored DNA samples extracted from soils collected for the GRDC National Paddock Survey (Lawes et al. 2018, GRDC project BWD00025). Soil pH had been measured in $CaCl_2$ by the GRDC project. Number of viable rhizobia/g of soil were calculated using correlations established between the qPCR results (gene copies/g soil) and the Most Probable Number (MPN) plant nodulation bioassay as described in Ballard et al. (2021).

Results
The qPCR tests for *Rlv* and *Mc* detected rhizobia in 465 and 340 of the 1091 national paddock survey samples, respectively. Detections of *Rlv* declined from 58% in soils with pH >6.5, to 30% in soils with pH <5.5. *Mc* detections also declined from 51% in soils with pH >6.5, to 12% in soils with pH <5.5.

The relationship between number of rhizobia and soil pH is shown in Figure 1. In soils where rhizobia were detected, mean Log_{10} number of *Rlv* declined from 3.7 in pH range 7.5 – 8.0, to 2.2 in pH range 4.0 – 4.5. The magnitude of decline for *Mc* was similar, although decreased numbers in the pH range 7.0 – 7.5 may indicate a more complex relationship at higher pH.

Ninety-six *Rlv* detections exceeded Log_{10} 4.0 when soil pH was >7.0, but there were no detections at this level when soil pH was <5.0. Fifty-eight *Mc* detections exceeded Log_{10} 4.0 when soil pH was >7.0. There were two instances where *Mc* detections exceeded Log_{10} 4.0 where soil pH was ≤pH 5.0, neither paddock had grown chickpea in the preceding two years.

The results presented in Figure 1 also provide some insight into the detection limits for the two qPCR tests. For the *Rlv* test this is about Log_{10} 1.7, and for the *Mc* test it is Log_{10} 2.0, with negligible detections below these levels.

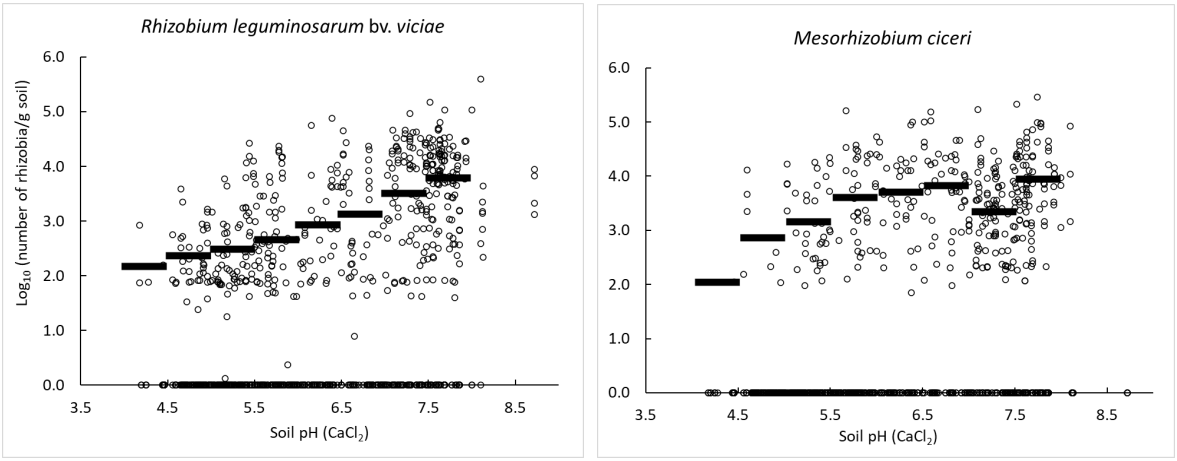

Figure 1. Effect of soil pH ($CaCl_2$) on the number of *Rhizobium leguminosarum* bv. *viciae* (left) and *Mesorhizobium ciceri* (right) in 1091 soil samples. Horizontal bars on each graph indicate the mean number of rhizobia/g soil in the eight half unit increments of pH between 4.0 and 8.0 (non-detections excluded from means).

Discussion

Specific qPCR tests were used to estimate *Rlv* and *Mc* number in all 1091 National Paddock Survey soil samples, the extent of which is impractical using the MPN nodulation test.

The results show that rhizobia numbers and percentage of soils with *Rlv* and *Mc* detections reduce with declining soil pH. Although some communities of *Mc* exceeded Log_{10} 4.0/g in soils below pH 5.5, there was a high level of non-detection (>85%) below pH 5.5. Paddocks identified with low pH (<5) and large rhizobia populations (>Log_{10} 3.0/g soil), especially after several years without a host crop, are potentially useful sources of acid tolerant strains.

The wide range of rhizobia levels at a given pH level illustrates that using soil pH alone to predict inoculation response is problematic. In this regard, the qPCR tests can be used to take the guess work

out of the need for rhizobia inoculation. Growers can access the rhizobia tests via PREDICTA® rNod (Ballard et al. 2021).

Acknowledgements

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POSTER SESSION

N_2 fixation of herbicide tolerant and non-tolerant pulse cultivars in response to grass and broadleaf herbicide application

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Key Words
Nitrogen fixation, nodulation, herbicide tolerance, imidazolinone

Introduction

With the increased prevalence of pulses in crop rotations has come an increased complexity in the range and frequency of herbicide groups being used for weed control. Herbicide residues and in-crop applications, particularly of those herbicides in Group 2 (B) (Imidazolinones and Sulfonylureas), can significantly reduce the growth, N_2 fixation and yield of intolerant legumes (Dear and Sandral 1999; Hollaway et al. 2006). In the last ten years, legume development programs have released imidazolinone herbicide tolerant cultivars of lentil, faba bean and field pea, which are now widely grown in Australia. In this paper we examine the nitrogen fixation capacity of tolerant cultivars.

Method

Five experiments were conducted to investigate the impact of commonly used grass and broadleaf weed herbicides on the nodulation and N_2 fixation of imidazolinone (IMI) tolerant and conventional (non-tolerant) pulse cultivars. The study was completed from 2019 to 2021 in medium and low rainfall cropping areas of south-eastern Australia. The experimental sites had populations of naturalised rhizobia and inoculation was not required. Grass and broadleaf weed herbicides were either not applied (control) or applied in accordance with common practice for each herbicide; either incorporated at sowing (IBS) or, applied post-sowing pre-emergent (PSPE) or, applied as a simulated soil residue (Table 1). All experiments had three replications and were established in randomised block designs or split plot designs with herbicide treatments in main plots and pulse cultivars in sub-plots. All plots received a pre-sowing knockdown herbicide (e.g. glyphosate + carfentrazone-ethyl) prior to sowing, followed by the experimental herbicide treatment. Nodulation was assessed at 12 weeks post sowing. Biomass and N_2 fixation (using the ^{15}N natural abundance method, Unkovich et al. 2008) were measured at mid-pod fill.

Results and discussion

The grass herbicide Propyzamide applied at label rate did not result in any significant negative effect on nodulation or shoot biomass on any cultivar (data not shown). Propyzamide reduced N_2 fixation of

assessed faba bean cultivars by 20% in one of two field trials (Table 1) whilst N_2 fixation of lentil and field pea was not significantly affected.

Broadleaf herbicide treatments imazethapyr and imazamox + imazapyr reduced the nodulation and biomass of conventional cultivars (PBA Jumbo2 and PBA Samira) compared to PBA Hallmark and PBA Bendoc, respectively (data not shown). Not surprisingly the amount of N_2 fixed of all conventional cultivars was reduced by imazamox + imazapyr, and by imazethapyr for lentil and bean (Table 1). In comparison, N_2 fixation for PBA Hallmark and PBA Bendoc was equal or greater than unsprayed controls and not significantly different for GIA Ourstar.

Table 1. Effect of four commonly used herbicides on the average amount of nitrogen fixed (kg shoot N/ha) by conventional and imidazolinone (IMI) herbicide tolerant cultivars of faba bean, lentil and field pea.

Herbicide name	Herbicide active	Application method	Faba bean		Lentil		Field pea	
			PBA Samira Conventional	PBA Bendoc IMI tolerant	PBA Jumbo2 Conventional	PBA Hallmark IMI tolerant	PBA Oura Conventional	GIA Ourstar IMI tolerant
Control	-	-	80_2	89_2	82_2	73_2	36_1	40_1
Propyzamide	Propyzamide	IBS	67_2	77_2	89_2	96_2	43_1	44_1
Spinnaker	Imazethapyr	PSPE	60_2^	91_2	33_2*	97_2	36_1	40_1
Terbyne	Terbuthulazine	IBS	87_2	95_2	82_2	70_2	41_1	36_1
Intercept	Imazamox + Imazapyr	#PSPE	62_2^	98_2	51_2*	97_2	28_1*	35_1

PBA =cultivar released by Pulse Breeding Australia. GIA= cultivar released by Grains Innovation Australia
# Applied PSPE to simulate a soil residue
$_{1, 2}$ subscripts indicate number of trials contributing to mean value
'*' indicates significantly less than unsprayed control (P<0.05), or '^' significantly less than unsprayed at one of two sites (P<0.05).

IMI-tolerant cultivars were on average able to fix up to 40 % more nitrogen than their conventional counterparts when exposed to IMI herbicides (PSPE -simulated residue). Importantly, no significant N_2 fixation or yield penalties were observed in the IMI-tolerant cultivars in the absence of herbicide treatment.

Summary

Herbicide tolerant pulses consistently show good nodulation, N_2-fixation, and grain yields when exposed to single applications of commonly used grass and broadleaf herbicides, compared to their conventional counterparts. This offers a useful tool for growers to manage weeds without compromising crop N_2 fixation. Further studies are needed to better understand impacts of multiple herbicide applications within a season, or when mixtures of commonly used grass and broadleaf weed herbicides are used.

Acknowledgements

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The role of Type III secretion system and effector protein NopP in host range determination within *Mesorhizobium ciceri* CC1192

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Key words
Mesorhizobium ciceri CC1192, chickpea, host range, Type III Secretion System, NopP

Abstract
Chickpea (*Cicer arietinum*) is the largest legume crop in Australia, grown mainly in northeastern and southeastern regions and parts of WA. Rhizobia are critical to the success of cultivation, and as such, inoculant strains with high N_2 fixing capability have been sourced internationally through selection programs. In the case of chickpea, *Mesorhizobium ciceri* CC1192, native to Israel, has been used as the commercial inoculant in Australia for more than 40 years (Hill et al., 2021). CC1192 is classified as having a narrow host range due to its inability to nodulate many other plants known to enter symbiosis with *Mesorhizobium* spp., including *Scorpiurus muricatus, Biserrula pelecinus* and members of the *Lotus* genus. In general, a narrow host range can be a desirable trait for commercial inoculants as it ensures that an inoculant does not ineffectively nodulate non-target legumes. For CC1192, the exact genetic factors restricting the CC1192 host range remain unknown.

Based on interrogation of the genome sequence, CC1192 appears to produce a highly undecorated Nod factor, with only *nodH* encoding a putative sulfyl transferase (Roche et al., 1991) present on the integrative and conjugative element (ICE*Mc*Sym^{1192}) . However, CC1192 also harbours a Type III Secretion System (T3SS) on this element, as well as a gene encoding a putative effector protein (*nopP*). T3SS and *nopP* have been shown to affect symbiosis in a host-specific manner in a range of rhizobia-legume symbioses (Deakin & Broughton, 2009; Sugawara et al., 2018).

To investigate whether the T3SS and/or *nopP* are involved in limiting the host range of CC1192, null mutants of the T3SS and *nopP* (locus tag: A4R28_21135) were generated by deleting the entire 20.9 kb T3SS region (including *nopP*) as well as a separate *nopP* deletion, using insertion-deletion mutagenesis, facilitated by suicide vectors which were generated via Gibson Assembly. The ability of these mutants to nodulate four hosts (*C. arietinum*, along with *S. muricatus, B. pelecinus* and *L. ornithopodioides*) will be compared to CC1192 in glasshouse experiments.

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Nodulation in *Ornithopus sativus* cv. Fran$_2$o is more sensitive to herbicides than plant growth

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Key words

Legumes, herbicide, serradella, nitrogen, dose response

Introduction and Aims

A staple of modern agriculture, nitrogen fertilisers are used globally and account for around 2% of the world's energy use (Sutton et al., 2013). In mixed farming rotations, legumes can be used as a biological means to supplement nitrogen, as well as providing high quality forage and protein for human consumption (Evers, 2011). A simultaneous increase in weed resistance to selective and non-selective herbicides has resulted in more complex weed control regimes in agriculture (Green, 2014). Researchers have investigated which mechanisms may be involved in the inhibition of the nitrogen fixation process by herbicides as detailed in Figure 1. However, little is known about the impacts of herbicides on nitrogen fixation within novel legumes. This project aims to explore interactions between popular herbicides and the ability of the novel legume *Ornithopus sativus* (French serradella cv. Fran$_2$o) to fix nitrogen.

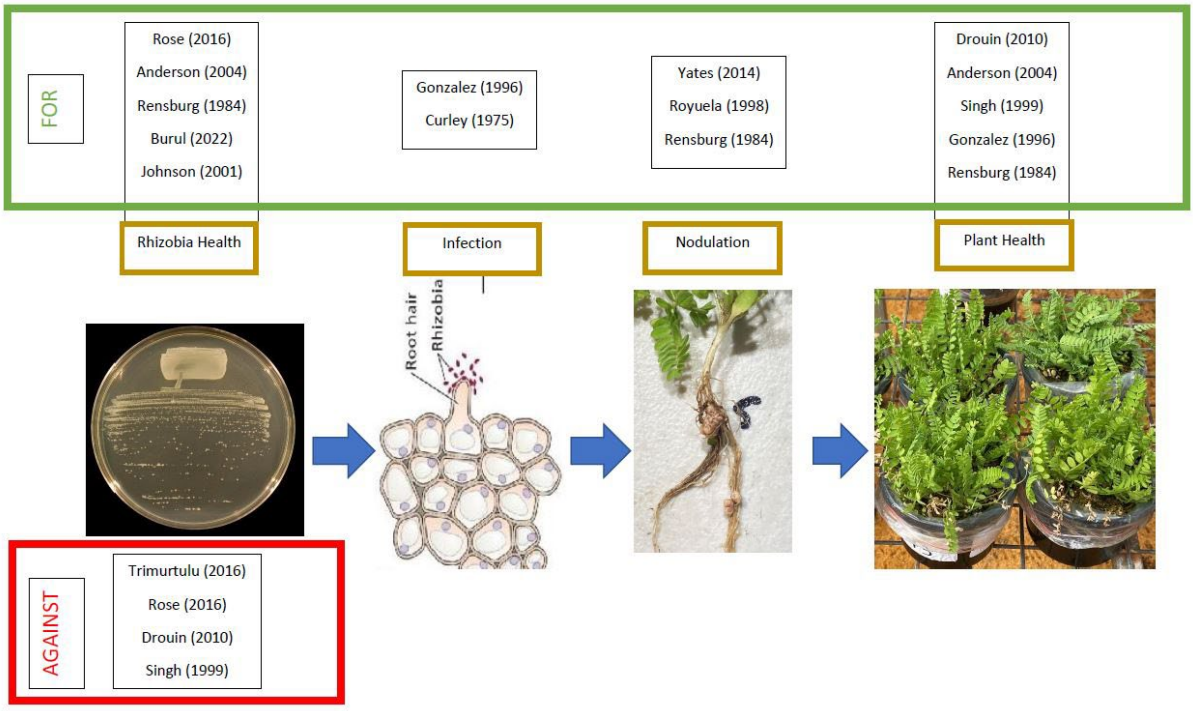

Figure 1. Proposals of authors as to where herbicides may disrupt the ability of a legume to nodulate.

Results

Fran$_2$o was grown in field trial and shade-house conditions in the presence of increasing rates of Diuron, Simazine and Imazethapyr, during which nodulation, root volume and plant dry weight were measured for herbicidal effects.

Results indicated discrepancies between the effect on top dry weight and nodulation (and therefore nitrogen fixing capability). Figure 2 shows the earlier toxicity of Diuron to root nodules before a later decrease in top dry weight as the herbicide rate increased. This toxicity pattern was also present in Simazine and Imazethapyr treatments.

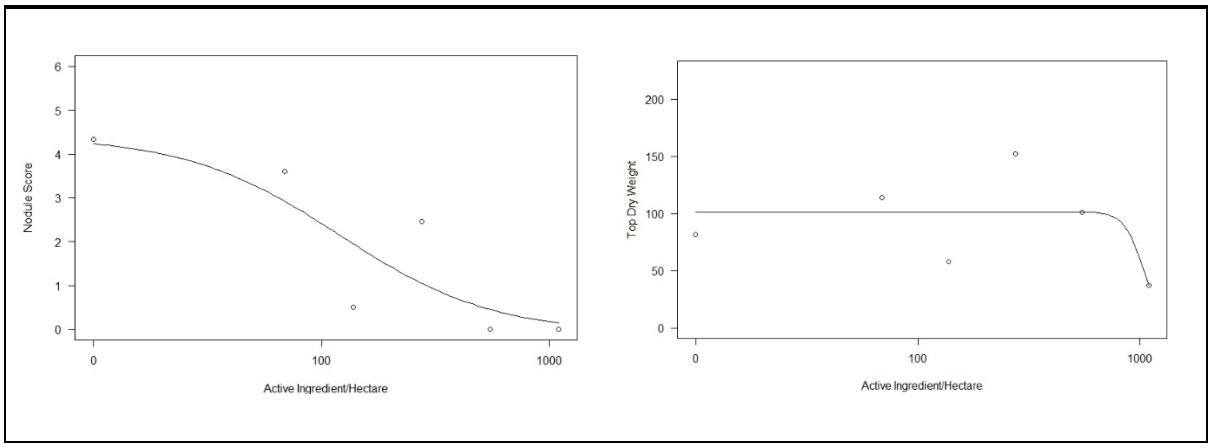

Figure 2. Dose response models of Fran$_2$o exposed to Diuron showing a decrease in the plant's nodulation before top dry weight increases.

Discussion

These results are evidence of herbicides interrupting a legume's ability to nodulate, separately from plant growth. The implication is that while farmers may observe a healthy legume paddock above the soil, their source of biological nitrogen has been significantly constrained. The apparent lack of nitrogen fixed by a seemingly 'healthy' legume crop may result in dissent and frustration with the competency of these agriculturally and ecologically valuable plants and may dissuade their use in farming environments. Further experimentation to identify where in the nodulation process (Figure 1) is vital to maintain the popularity of legumes in farming rotations.

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Epigenetic control of bacterial quorum sensing and horizontal DNA transfer

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Keywords

Epigenetics, quorum sensing, horizontal DNA transfer, Mesorhizobium

Abstract

Mesorhizobium japonicum R7A carries an integrative and conjugative element (ICE) named ICE*Ml*Sym^{R7A}, which harbours genes for nitrogen-fixing symbiosis with *Lotus* legumes (Sullivan et al., 1995). Here we describe how R7A controls the rate of ICE*Ml*Sym^{R7A} transfer through a complex network of auto-regulating DNA-bind proteins and cell-cell communication called quorum sensing (QS).

Bistable gene regulation can enable genetically identical bacteria to differentiate into phenotypically distinct populations (Veening et al., 2008). We discovered that a subpopulation of R7A cells differentiate into high-frequency ICE donors that produce QS signaling molecules called *N*-acyl-homoserine lactones (AHLs). These cells, termed R7A*, emerged from ~2% of colonies derived from R7A populations. R7A* cells maintained this phenotype through serial laboratory passaging and even following plant symbiosis. Extensive genome sequencing failed to identify genetic changes responsible, suggesting the state is maintained epigenetically. The R7A* state was not transferred with ICE*Ml*Sym^{R7A} following transfer to an isogenic non-symbiotic recipient, further supporting the hypothesis that R7A* is epigenetically maintained (Ramsay et al., 2022).

R7A ICE transfer is normally repressed by the QS antiactivator QseM, and transcription of *qseM* is controlled by a regulatory DNA-binding protein QseC (Ramsay et al., 2013). Here, transcriptome sequencing revealed *qseM* transcription was abolished in R7A*; furthermore, an RNA transcript antisense to *qseC* was present in R7A but not R7A*. Deletion of the antisense promoter converted R7A cells into an R7A*-like state. A second adjacently-encoded DNA-binding protein QseC2 repressed the anti-*qseC* transcription. Interestingly, QseC2 overexpression from a plasmid stimulated cells to enter the R7A* state and they remained in this state even after curing of the overexpression plasmid (Ramsay et al., 2022).

DNA-binding assays, mutagenesis and transcriptional fusions indicate R7A* establishment requires the accumulation of both QseC2 and QseC in cells, which results in *qseM* repression and activation of quorum sensing, which primes ICE*Ml*Sym^{R7A} for horizontal transfer (Ramsay et al., 2022).

Like other bistable systems, the described ICE*Ml*Sym^{R7A} switch may accomplish a bet-hedging strategy in which the ICE partitions a small host subpopulation to prepare for DNA transfer, sparing the majority of the host population from the fitness costs involved in horizontal transfer. This system seems unique however in that it enables persistent vertical inheritance of the transfer-primed state in R7A*. These discoveries provide novel insights into strategies employed by mobile elements to optimize horizontal transfer rates in the face of potential host impacts.

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The role of ICESym in symbiotic performance of chickpea mesorhizobia

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Key words

Mesorhizobium, chickpea, symbiosis island, ICESym, symbiotic performance

Abstract

Genetic diversity of mesorhizobia in soil arises from horizontal gene transfer of symbiosis islands which are integrative conjugative elements (ICEs). Symbiotic ICEs (ICESyms) carry nodulation and nitrogen fixing genes which are transferred to non-nodulating mesorhizobia in soil affording them the ability to nodulate legume hosts. ICESyms have been characterised in mesorhizobia from *Lotus corniculatus* and *Biserrula pelecinus* as tripartite mobile genetic elements, that when transferred to resident soil mesorhizobia, frequently give rise to poorly effective strains (Sullivan and Ronson 1998; Nandasena et al. 2007; Haskett et al., 2016).

ICESyms in chickpea mesorhizobia may be tripartite or monopartite (Greenlon et al., 2019). Hill et al. (2021) demonstrated that novel chickpea isolates from soils in NNSW had arisen from transfer of the symbiosis island from CC1192 (ICE*Mc*Sym^{1192}) to diverse non-nodulating soil mesorhizobia. However, novel chickpea strains isolated from Australian soils show little variation in symbiotic performance (Hill et al., 2021; Zaw et al., 2021). Hill et al. (2021) demonstrated that ICE*Mc*Sym^{1192} could be transferred to an ICE-cured derivative of *M. japonicum* R7A known as R7ANS and that the symbiotic performance of exconjugants did not differ significantly from the donor CC1192 thus concluding that ICE*Mc*Sym^{1192} confers effective symbiotic potential to non-nodulating recipient strains.

A further five genetically distinct monopartite and tripartite ICESyms in chickpea nodulating mesorhizobia were characterised in the SUNFix culture collection, with several theoretically capable of transfer. In addition, one of the strains was found to be genetically indistinct from CC1192 but devoid of its ICESym which should serve as a useful recipient to study the contribution of different chickpea nodulating ICESyms to symbiotic performance of exconjugants. The aim of this research is to investigate the role of diverse ICESyms in the symbiotic performance of chickpea mesorhizobia.

Inoculation of chickpea hosts (cv. Kyabra) with strains carrying genetically diverse ICESyms indicated variation in symbiotic performance and confirmed the absence of symbiosis with strains lacking functional ICESyms. Further assessment of these strains across a genetically diverse set of chickpea cultivars indicated that mesorhizobial strains rather than plant hosts were responsible for most of the variation in symbiotic performance. Consistent with the findings of Hill et al. (2021), ICESyms of the three sequenced Australian field isolates in the SUNFix collection shared identical (>99.9%) homology

with the commercial inoculant strain CC1192 and symbiotic performance of 113 field isolates varied but was not significantly different to CC1192.

Conjugation experiments between mesorhizobial donor strains and two different non-nodulating soil communities indicated variation in overall symbiotic performance of soil communities after inoculation with strains carrying different ICESyms. Specifically, introduction of SU1828 to soil communities resulted in a 9% overall gain in shoot dry weight of chickpea (cv. Kyabra) which was a significant increase compared to the commercial inoculant strain. A number of individual nodule isolates from these experiments with diverse RP01 fingerprints exhibited higher symbiotic performance than donor strains in separate inoculation experiments. These isolates are currently being analysed to confirm transfer of ICESyms from donor strains. Conjugation experiments to transfer diverse ICESyms to ICE-cured recipient strain SU3069 are also currently underway.

Our results along with those of Hill et al. (2021) support the hypothesis that ICE*Mc*Sym^{1192} is likely to be the only ICESym found in chickpea nodules in Australian soils since CC1192 has been the only commercial inoculant strain for chickpea since commercial production expanded in the 1970s, hence symbiotic performance may be limited. Our results concur with published studies that symbiotic performance of chickpea mesorhizobia isolated from Australian soils do not vary significantly from the commercial strain and thus there is little risk that transfer of ICE*Mc*Sym^{1192} reduces effectiveness of soil communities. However, introduction of a new ICESym may lift the overall performance of naturalised soil populations. Hence further work will be carried out to examine the potential for different ICESyms to increase symbiotic performance of soil mesorhizobial populations in Australian chickpea growing regions.

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Investigation of symbiotic mobility and horizontal gene transfer in soybean rhizobia

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Keywords

Bradyrhizobium spp. mobile genetic elements, novel microsymbionts, soybean

Abstract

Soybean (*Glycine max*) is one of the main agricultural crops in Brazil, due in large part to a well-organised inoculation strategy, with a range of highly efficient inoculant strains (Hungria, 2011). In contrast, soybean production in Australia is much lower, but the same strain (CB1809) has been in use to inoculate this grain legume since the 1960s (Bullard, 2005). Native rhizobia in both countries appear unable to nodulate soybean, however in Brazil there are now several reports of rhizobia genetically distinct to inoculant strains being isolated from inoculated soybean. How they evolved and how efficiently they fix N_2 is currently unknown. In Australia, there has been no recent genetic investigations into the diversity of soybean nodulating rhizobia in areas of cultivation of this grain legume. Therefore, this study will assess the genetic diversity and efficiency of soybean nodulating rhizobia sampled from cultivated soybean from both Brazil and Australia.

Several strains isolated from Brazil as well as strains trapped and/or isolated from soybean-growing areas of Australia will first be authenticated and then genotyped. Isolates will be grouped based on their RAPD fingerprint pattern and new isolates that differ phenotypically and genotypically from the inoculant strain will be chosen for whole-genome sequencing using the Illumina platform. The genomes of the new sequenced lines will be described to identify the origin of the symbiosis genes, through the comparison of well-characterized *Bradyrhizobium* lines and the recently completed genomic sequence of the CB1809 soybean inoculant genome, as well as USDA110 (Kaneko, 2002) and USDA122 (Sugawara, 2017), with the chromosomal background characterized using a custom script developed by Colombi et al. (2021). The investigation of symbiotic genes will be conducted by extracting symbiotic genes and generating data for further genomic analysis. The genomic analysis will be complemented with a glasshouse-based evaluation of the symbiotic N_2-fixation efficacy of the new strains. From these data, the impact of horizontal symbiotic gene transfer on the efficiency of N_2-fixation will be determined.

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Lebeckia spp. as a plant-based solution for soil amelioration in Western Australian deep sandy soils

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Keywords

Sandy soils, low carbon, nutrient cycling, farming systems

Abstract

In the Wheatbelt region of Western Australia, challenges such as soil depletion and nutrient leaching of infertile deep, sandy soils reduce agricultural productivity. Agricultural management practises, which include summer fallow, are increasing wind erosion risk and depleting soil carbon. Due to their low reactive surface area, these substrates have a limited capacity to supply water and nutrients to plant roots, presenting a core limitation to crop productivity. New approaches such as using perennial, ley-farming and innovative cropping system options could ameliorate deep sands by raising soil organic matter and fertility and stabilising soil through increased crop cover, below-ground biomass and microbial activities. The introduction of perennial deep-rooted legumes in these farming systems could prevent degradation and ameliorate these soils, limiting the amount of chemical inputs through fertilisation and creating revenue from grazing. Between planting perennial legumes, *Lebeckia ambigua*, a species adapted to the sandy soil of South Africa, could be a solution to overcome these challenges and provide a grazing summer option for mixed farming in Western Australia. However, *Lebeckia ambigua* is a wild plant that needs further investigation before widespread use in the Western Australian wheatbelt. The national Cooperative Research Centre for High Performance Soils (Soil CRC) and Murdoch University are investigating the performance and benefit of introducing *Lebeckia* spp. in local farming systems with deep sandy soils where the above challenges limit farming production.

Factors governing attachment of *Rhizobium leguminosarum* to legume roots

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Key words
Rhizobia, pea, roots, bacterial attachment, pH

Abstract
Primary attachment of rhizobia to host legume roots, the first physical interaction during symbiosis, depends on pH. Parsons et al. (submitted) used genome-wide insertion sequencing (INSeq) together with luminescence-based attachment assays to demonstrate that primary attachment of *Rhizobium leguminosarum* biovar *viciae* 3841 to *Pisum sativum* (pea) roots is more complex than previously thought. In total, 115 genes are needed for initial attachment under one or more test conditions (acid, neutral or alkaline pH), with 22 required under all conditions (Figure 1). These include those encoding a cell-surface filamentous hemagglutinin adhesin (RL4382) and its transporter (RL4381), transmembrane protein RL2400, RL3752 (PssA, glycosyl transferase) affecting capsular polysaccharide and transcriptional regulator RL4145 (PckR). The 54 genes required for attachment at pH 7.0 were investigated for the effect their mutation has on the ability to form a nitrogen-fixing nodule.

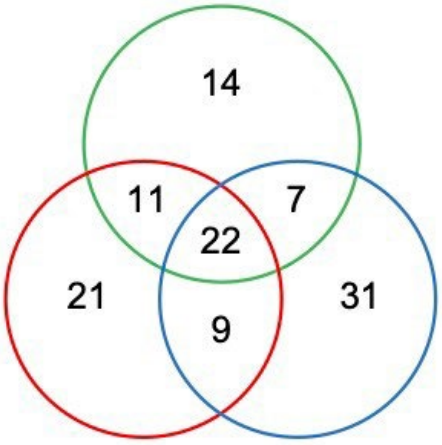
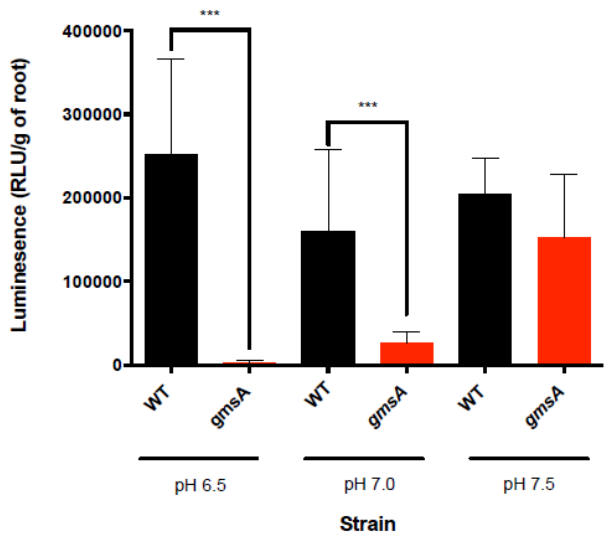


Figure 1. Rlv3841 genes required for primary attachment to pea roots under acidic, neutral and alkaline pH. A total of 115 genes were classified as required for attachment in INSeq experiments performed at pH 6.5, pH 7.0 and pH 7.5, with 22 genes being required for attachment at all pHs. Colour of circle indicates pH; on the left-hand side (red) = pH 6.5, at the top (green) = pH 7.0 and on the right (blue) = pH 7.5.

Unsurprisingly, the bacterial cell surface plays a key role in attachment to roots, but genetic requirements are not necessarily constant and are affected by environmental conditions.

Glucomannan biosynthesis protein A (GmsA, RL1661) is required to attach to roots at pH 6.5 but not at pH 7.5 (Williams et al., 2008). We show by INSeq and attachment assays using Lux-labelled bacteria, requirement for *gmsA* at pH 6.5. and pH 7.0, while it is non-essential at pH 7.5 (Figure 2).

A)



B)

Gene	INSeq classification			Primary root attachment (%age WT) of strain with gene mutated		
	pH 6.5	**pH 7.0**	**pH 7.5**	**pH 6.5**	**pH 7.0**	**pH 7.5**
RL1661 (*gmsA*)	DE	DE	NE	1%*	16%*	71%

Figure 2. A) Effect of pH on primary attachment to pea roots. Attachment of Rlv3841 (WT) (black) and strain mutated in *gmsA* (RL1661) (red) at acidic, neutral and alkaline pH using the Lux-based whole root attachment assay. Luminescence (RLU/g of root) shows bacterial attachment after 1 h. n = 10. Data are expressed as the mean ± SD. ***= $p < 0.001$ using Student's t-test. There is no statistically significant difference between primary attachment to roots of Rlv3841 (WT) at different pHs. **B) INSeq classification of RL1661 (*gmsA*) and attachment of strain mutated in RL1661 (*gmsA*), at different pHs.** Results expressed as mean root attachment of test strain in percent, indexed to 100% attachment for WT (Rlv3841) under each condition. Asterix signifies statistically significant difference from WT using an unpaired t-test *= $p < 0.0005$.

Our results demonstrate the complexity of primary root attachment and diversity of mechanisms involved in the initial reaction between bacteria and plant roots on the pathway to successful symbiosis.

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Clade-dependent effects of drought on nitrogen fixation and its components – number, size, and activity of nodules in legumes

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Key words

Amide, plasticity, senescence, symbiosis, trade-off

Introduction

Drought affects the growth of legumes directly, and indirectly, by reducing total nitrogen fixation (Duc et al., 2015). We compiled published data to assess the sensitivity to water deficit of plant growth and total nitrogen fixation traits i.e. the number of nodules per plant, average nodule mass, and nitrogen fixation per unit nodule mass. Hierarchies of phenotypic plasticity have been established for seeds and organelles, whereby the high plasticity of seed number is associated with the stability of seed size (Divito and Sadras, 2014). By analogy, our first hypothesis is that there is a hierarchy of plasticities between nitrogen fixation traits. Our second hypothesis is that determinate nodules are more sensitive to water deficit than their indeterminate counterparts, because the latter can reactivate meristems when water becomes available.

Results

In our sample of data, onset of stress treatment averaged 28 d after sowing; median duration of stress was 12 d; and intensity of stress (ratio of shoot biomass between stressed and control) averaged 0.65. Total nodule mass and total nitrogen fixation were more sensitive to water deficit than shoot mass (Figure 1). Nodule number per plant was more sensitive to water deficit than both average nodule mass and nitrogen fixation per unit nodule mass. Average nodule mass and nitrogen fixation per unit nodule mass had similar sensitivity to water deficit. Water deficit affected determinate (ureide exporting) nodules more severely than indeterminate (amide exporting) nodules (Figure 2).

Discussion

Water deficit reduced total nitrogen fixation and nodule mass more severely than shoot mass. A similar hierarchy of plasticities was found for deficit of phosphorus, sulphur or potassium that reduced nodule mass more severely than shoot mass in grain and forage legumes (Divito and Sadras, 2014). This differential sensitivity is consistent with higher antioxidant enzymatic activity (Mouradi et al., 2018) and higher accumulation of sucrose and amino acids (Soba et al., 2019) in nodules compared with leaves, in response to water deficit.

Shoot mass, the number of nodules and total nitrogen fixation were more responsive to water deficit in the Millettioids (ureide exporters, determinate nodules) than in IRLC legumes (amide exporters, indeterminate nodules). Plants have an abundance of reduced carbon under water deficit (Muller et al., 2011), and the differential sensitivity we identified may correlate with more consumption of

carbon for bacteroids in determinate nodules where bacteroids typically retain the ability to reproduce, as opposed to those in indeterminate nodules (Denison, 2000). In conclusion, drought at early development stages affects determinate nodules more severely than indeterminate nodules and elicits a hierarchy of plasticities among nitrogen fixation traits.

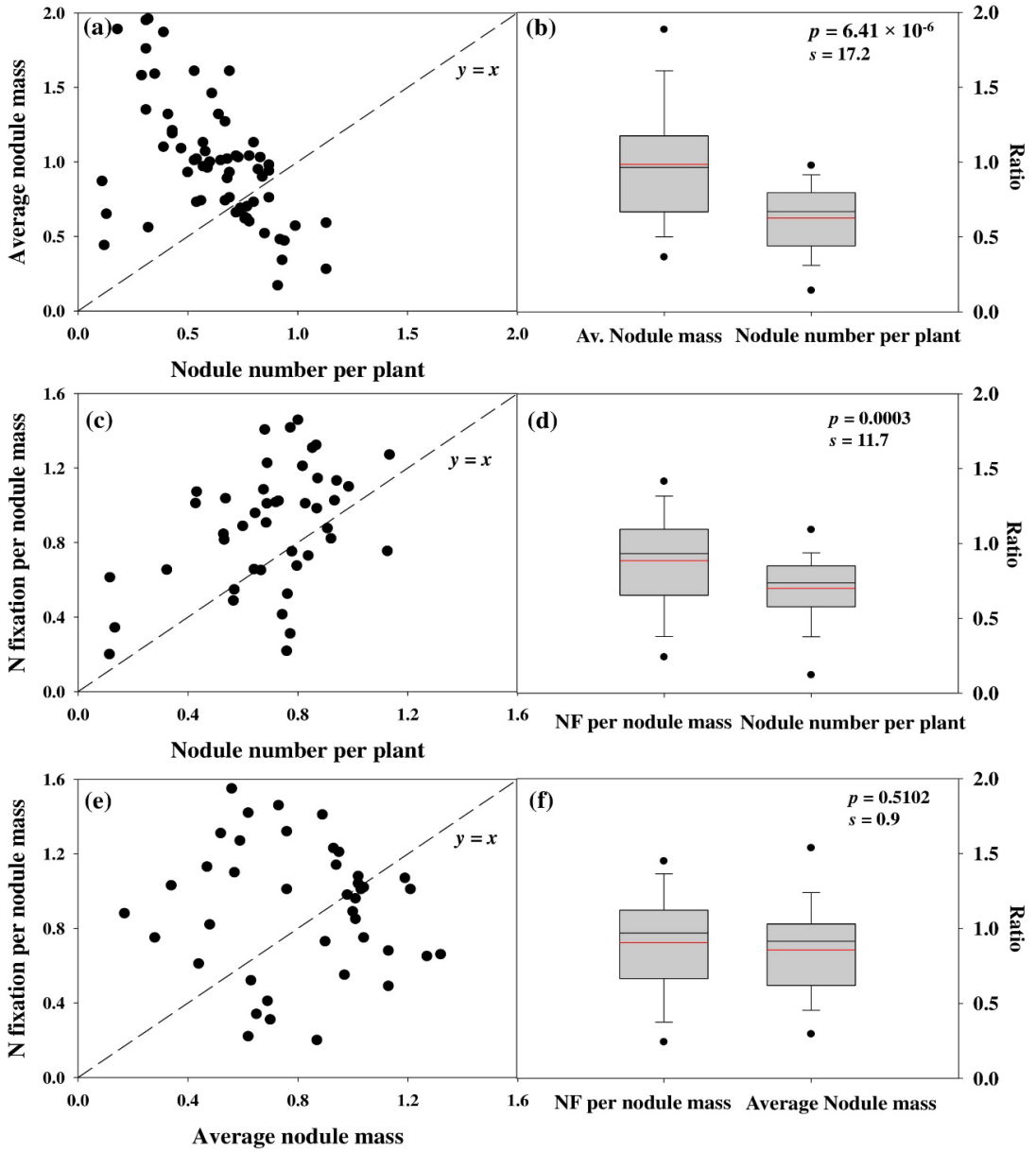

Figure 1. Comparison of (a, b) average nodule mass and nodule number per plant; (c, d) nitrogen fixation per nodule mass and nodule number per plant; and (e, f) nitrogen fixation per nodule mass and average nodule mass in response to water deficit. The y = x line is the null hypothesis of traits with similar response to water deficit. In (b-f), ratios close to 1 indicate less sensitivity to water deficit; p and s are from paired t-test. In box plots, red lines are means, lines are 25th, 50th and 75th percentiles, whiskers are 10th and 90th percentiles and dots are 5th and 95th percentiles.

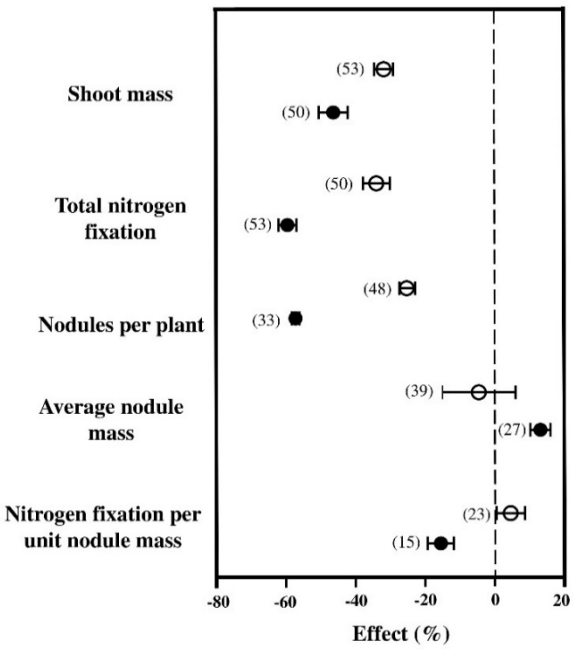

Figure 2. Clade-dependent effects of water deficit on plant shoot mass and nitrogen fixation traits. Clades are Millettioids, ureide transporters with determinate nodules (closed symbols) and IRLC, amide transporters with indeterminate nodules (open symbols).

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Source to sink nitrogen partitioning and the role of ureides and amides in chickpea

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Key words
Nitrogen Fixation, ureides, amides, nodules, chickpea

Abstract
Symbiotic temperate legumes such as chickpea (*Cicer arietinum*) harness atmospheric nitrogen via root nodules, formed through a symbiotic partnership with nitrogen-fixing rhizobia. Nodules in legumes are responsible for converting fixed N into a form usable by the plant for long distance transport and storage. This consists of ureides or amides, typically synthesised in the nodules of tropical and temperature legumes, respectively. Nodule morphogenesis in the form of determinate and indeterminate structures have also been known to indicate the synthesis of either ureides and amides. Chickpea, a temperate legume, has received little attention to determine which N-assimilation pathway predominates in its nodules and the key transporters responsible for source to sink N transport. Reports in the literature are contradictory, prompting us to re-examine this issue.

Though a colorimetric assay and mass spectrometry, the levels of ureides and amides were measured in leaf, root and nodule tissue during growth experiments in sand under low N conditions (0.5 mM KNO_3) and harvested at 5-day intervals over 10-35 DAI (Days After Inoculation) (Figure 1) (Collier and Tegeder, 2014). Differences in levels of ureides and amides were found in chickpea compared to soybean, a typical ureide predominant legume. Particularly, total ureide levels were significantly higher is all tissues at every timepoint measured in soybean compared to chickpea. The same tissue was harvested for RT-QPCR of N transporters and metabolism genes, where *GmPur1*, the first enzymatic step in ureide synthesis was identified as a key indication of ureide production (Figure 2) (Smith and Atkins, 2002). Fold increases of *GmPur1* expression in soybean nodules far exceeded levels seen in the equivalent *CaPur1* in chickpea nodules. Key N metabolism and nodule/root transporter genes were identified in chickpea (Slasher) though mRNA sequencing of nodule and root tissue harvested during pre-fixing and peak-fixing timepoints. Together, these results indicate that amide production and transport in chickpea exceeds that of ureides and identify key genes that function in the export of amides out of the nodules. This may provide an avenue for increasing N-fixation and source to sink transport of amides in chickpea through genetic manipulation.

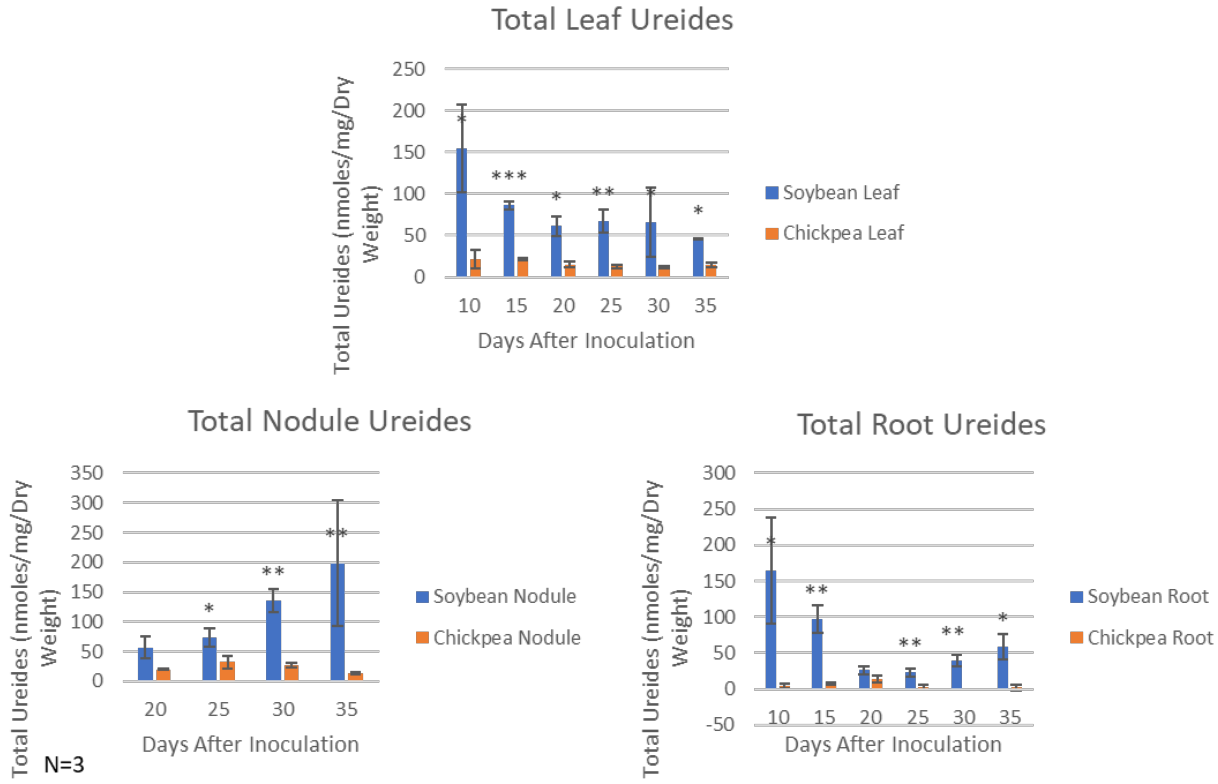

Figure 1. Total Ureides includes both Allantoin and Allantoic Acid. Soybean and Slasher grown in sand an inoculated with rhizobia at sowing. Leaf, root and nodule tissue harvested in triplicates at 5-day intervals (10 DAI – 35 DAI). Tissue lyophilized for 3 days through freeze drying and ground into a fine powder prior to analysis. Asterisks indicate significant differences by One-Way ANOVA test of total ureides from soybean (*P<0.05, **P<0.01, ***P<0.001). Ureide assay adapted from Collier and Tegeder (2012).

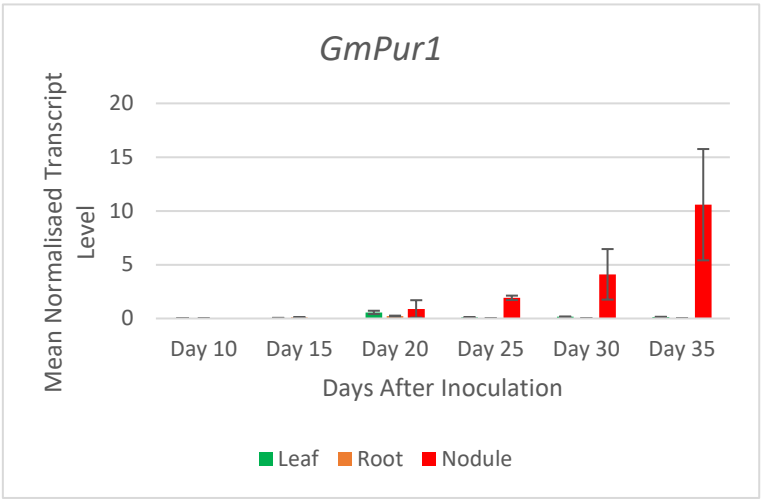

Figure 2. Soybean transcript data normalised against *GmACT11 and GmELF1B.*

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Evolution of symbiotically effective rhizobium strains for North American cultivars of dry bean

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Key words
Rhizobium, evolution, NGS, dry beans

Introduction

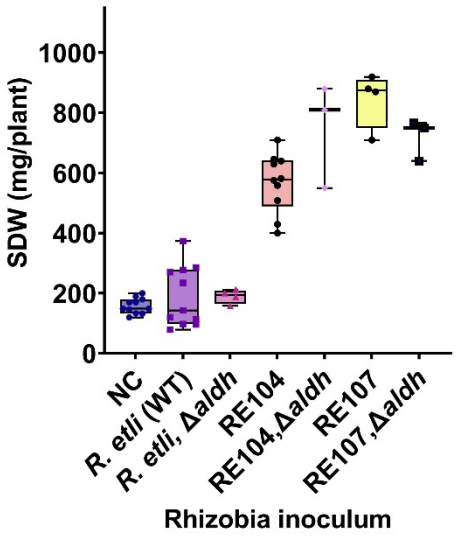
Global production of the pulse crop *Phaseolus vulgaris*, commonly known as dry bean, is estimated at 30 million tonnes annually. Canada is a major producer of many pulse crops and contributes 360 thousand tonnes of dry bean to global production annually. However, commercial bacterial inoculants for biological nitrogen fixation of dry beans in North America either form very poor nitrogen fixing relationships or do not fix nitrogen altogether. Due to this, bacterial inoculants generally are not used on dry bean crops, and instead favour application of artificially produced nitrogen fertilizers. *Rhizobium etli* CFN42 can form a nitrogen fixing symbiotic relationship with some cultivars of *P. vulgaris* but fail to fix nitrogen with North American strains. Previous studies have shown that adaptive evolution of rhizobia using legume plants as a selective pressure could lead to improvements in nitrogen fixation[1,2]. This led us to the hypothesis that CFN42 could be evolved during symbiosis with *P. vulgaris* in nitrogen deficient conditions to promote selection of mutations which result in biological nitrogen fixation.

Results

Initial inoculation of *P. vulgaris* with CFN42 resulted in yellow chlorotic plants with small white nodules along the roots. Bacteria were isolated from three separate nodules to form three distinct lineages, and then reinoculated onto new plants. Isolation and reinoculation was repeated eight times for each lineage. After two reinoculations a nitrogen fixing symbiotic relationship in all lineages was observed. Plants showed strong growth with 2-4x the shoot dry weight compared to when inoculated with wild-type CFN42, had green leaves and exhibited nodulation along the root crown. In addition, nodulation was observed to occur faster in evolved strains once nitrogen fixing symbiosis was achieved. In order to determine what mutations may have resulted in these changes, the genomes of all strains were isolated and sequenced using a Nanopore MinION. The wildtype genome of CFN42 was assembled using Flye, and each genome from each lineage was mapped to this assembled genome using Minimap2 followed by variant calling.

A fusion between the B and D plasmids could sometimes be observed, but this was random and not found to be linked to gain or loss of nitrogen fixation. Otherwise, no major changes in genome architecture were observed suggesting gain of nitrogen fixation was likely due to point mutations or smaller insertions/deletions.

The data was analysed for mutations which became conserved within the lineages after they appeared. We have focused on two mutations. These are a frameshift which restores the reading frame of a gene annotated as *gmd*, and a point mutation which abolishes the start site of a putative aldehyde dehydrogenase. The mutation in *gmd* is present in all strains and lineages and always occurred first. The mutation in the aldehyde dehydrogenase occurred in two of the three lineages. Work is ongoing to confirm the *gmd* mutation is necessary to restore a nitrogen fixing symbiosis. The loss of the start codon for *aldH* was correlated with a 15% increase in measured dry weights during lineage isolation (RE107). When a deletion of the entire aldehyde dehydrogenase gene was created and introduced into a strain carrying an initial *gmd* mutation it was observed that dry weights increased and were like those found when plants were inoculated with RE107.



Discussion

Overall, our work is a proof of concept that laboratory direct evolution can be used for the development of a symbiotically effective inoculant strain. We have identified two mutations which when present in our lab strain of CFN42 allow for nitrogen fixation to occur when used as an inoculum for Canadian cultivars of dry bean and improve upon the yield. Ideally, we would like to use this practice of directed evolution to create a competitive inoculum strain that would survive in Canadian soils. To this end we have isolated 6 strains of rhizobia native to soil in Manitoba[3] and are currently in the process of carrying out directed evolution studies.

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Whole genome sequencing of commercial rhizobia reveals potential novel species

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Keywords

Commercial rhizobia, nitrogen fixation, whole genomes

Abstract

Australia's commercial legume inoculant industry is a gold standard world-wide as there is a guiding principle of one commercial rhizobial strain per legume host group. Introduction of a new commercial strain targeting major legume hosts is a stringent evidence-based process that requires a minimum of ten field experiments over two seasons before adoption to the Australian market. As a result of these criteria there is a low rate of strain change.

Despite being taxonomically similar, most of Australia's commercial rhizobia have specific legume host ranges. To date the taxonomy of most of the commercial strain collection has not been clearly elucidated.

Whole genome sequencing of 34 commercial rhizobial strains from the Australian Inoculants Research Group collection was conducted. A total of ~1300 GB of combined short read (Ion Torrent) and long read (MinION) sequencing data was produced. This equates to an average of over 200-fold sequence coverage for each strain's genome. Long read genome sequencing data (MinIon) was demultiplexed with Porechop v0.2.4 and then assembled with Trycycler v0.3.0 using triplicate sequencing subsets of fifty times depth. Long read assemblies were used as a scaffold and polished with medaka v1.1.2 and five rounds of Pilon v1.23 polishing using short read data from an IonTorrent S5. Average nucleotide identity (ANI) was calculated between 34 genome sequences in this study and a reference list including 2,072 genomes of *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Azorhizobium* and *Georhizobium* from NCBI using FastANI v1.31 within this study as the query list.

Several of the Australian commercial strains had less than <96% match to any of the 2000+ references; these include the rhizobial strains WSM1592 for sulla, CB82 for stylo, CB1650 for Caribbean stylo (Verano), CIAT3101 for pinto peanut and CB2312 for jointvetch indicating the potential for these to be novel species. This finding warrants further investigation.

Mesorhizobium SyrA is a symbiotic regulator of exopolysaccharide biosynthesis and affects nodulation of *Lotus japonicus* Gifu

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Key words

Mesorhizobium, nitrogen fixing, symbiosis, host-range, exopolysaccharide

Abstract

Rhizobia are a group of soil bacteria that form nitrogen-fixing symbioses with legumes through the formation of root nodules. The *Mesorhizobium-Lotus* symbiosis is one model used to study how these relationships are established. *Mesorhizobium* spp. acquire genes necessary for symbiosis through horizontal transfer of integrative and conjugative elements (ICEs) or symbiosis islands. Differences in the genes carried on these ICEs contribute to differences in the host-range and effectiveness of the symbiosis with *Lotus* spp. *Mesorhizobium* spp. strains can be classified into two host-range groups based on their ability to nodulate *Lotus pedunculatus*. Group I strains (e.g. *M. japonicum* R7A) only induce uninfected nodule primordia on this host, while Group II strains (e.g. *M. jarvisii* NZP2037) efficiently induce effective nodules. Both groups can effectively nodulate *Lotus japonicus* Gifu.

Comparative analyses revealed a second copy of *syrA* on the NZP2037 island that is not present on the R7A island. This copy has been translocated downstream of *nodB* and is likely expressed during early symbiosis by NodD in response to plant flavonoids. In *Sinorhizobium meliloti*, *syrA* encodes a small cytoplasmic membrane protein that upregulates exopolysaccharide (EPS) production. In contrast, we show that NZP2037 SyrA acts as a repressor of EPS production and when expressed during early symbiosis it reduces the nodulation efficiency of the strain on *L. japonicus* Gifu, including on plant mutants that lack the EPR3 EPS receptor. Furthermore, R7A harbours five copies of SyrA-like proteins (ExoX's) which all repress EPS production to various levels and have been identified to interact with the galactosyltransferase, ExoY. Together these results indicate that rhizobial EPS is a highly regulated signalling molecule that plays an important role in the early steps of nodulation of *L. japonicus* Gifu independently of its recognition by EPR3.

Understanding Plant Colonisation and Nitrogen Fixation by Gammaproteobacteria Using Metabolic Modelling and Multi-omics Approaches.

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Key words

Plant Growth Promotion, Gammaproteobacteria, omics data, colonisation

Abstract

As pressure grows to increase agricultural productivity in a sustainable way to meet future food demands, the plant-associated microbiota have gained ever-increasing attention due to their significant potential to increase sustainable agricultural production. These diverse microbes interacting with themselves and with the plants, form a key part of the rich ecosystem of crops, and can be harnessed to aid with nitrogen fixation, nutrient acquisition and to improve plant defences (Berendsen et al., 2012; Philippot et al., 2013; Wei et al., 2015). One of the major aims in biological research in the 21st century is expanding the use of biological nitrogen fixation to a wider range of crop plants such as cereals. The use of Gammaproteobacteria as plant growth promoting bacteria (PGPB) offers a promising opportunity in this regard. However, to be able to successfully utilise PGPB inocula to increase crop yields with consistent results, it is crucial to have a clear understanding of their root colonisation (Haskett et al., 2021).

In this study, high-throughput multi-omics data and genome-scale metabolic models (GEMs) are used to gain mechanistic understanding of the key colonisers *Kosakonia radicincitans* DSM16656^T (KrDSM16656) and *Enterobacter ludwigii* AA4 (ElAA4). Both of these strains are Gammaproteobacteria, strong colonisers and are of notable interest for plant growth promotion. The omics and *in silico* analysis is centred around a niche-specific framework, to decipher the metabolic differences and cellular reprogramming of the bacteria during root colonisation of barley (*Hordeum vulgare*) (Figure 1). This systematic and GEM-based approach with the acquired omics data not only provides beneficial information on the colonisation of the strains but can also provide insights into the plant growth promoting abilities of the bacteria. Of particular interest in this regard is to use this environmental understanding and characterisation of the diazotrophic capabilities of KrDSM16656 to gain insights into the necessary reprogramming of the multi-layered regulatory control of nitrogen fixation to enable targeted root-associated expression.

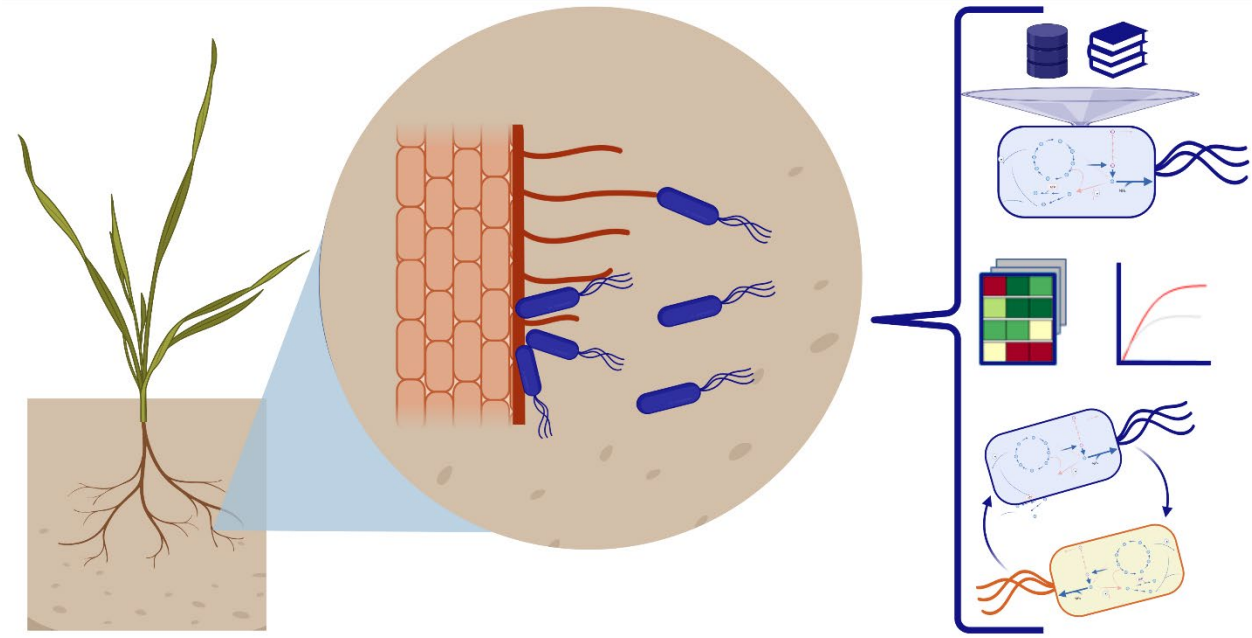

Figure 1. Niche-specific framework to decipher the metabolic differences and cellular reprogramming of the bacteria of interest during root colonisation of barley (*Hordeum vulgare*). Genome scale metabolic models (GEMs) are constructed for these strains and used along with high-throughput multi-omics data to gain a mechanistic understanding of their behaviour. The GEMs can be integrated with the omics data and with literature values to generate condition-specific models, used iteratively along with targeted experimental work, or applied in a complementary manner as a part of larger modelling frameworks. Created with BioRender.com.

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Oxygen regulation of symbiotic nitrogen fixation in *Mesorhizobium ciceri* CC1192

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Key words
Oxygen, regulation, symbiotic, nitrogen fixation

Abstract
Oxygen (O_2) concentration inside legume root nodules is tightly controlled in order to allow nitrogenase to function. While nitrogenase requires the near anoxic conditions facilitated by the nodule to function, rhizobia are provided enough O_2 within the nitrogen fixing zone of the nodule to respire via the cytochrome cbb3 terminal oxidase, encoded by *fixNOQP*, which fuels nitrogen (N_2) fixation (Delgado et al., 1998). While O_2 tension controls nitrogen fixation broadly, there are large variations in the regulatory networks that different rhizobia employ. To date most work on oxygen regulation of symbiotic nitrogen fixation has focussed on species such as *Bradyrhizobium japonicum*, *Sinorhizobium meliloti*, and both *Rhizobium leguminosarum* and *R. etli* CFN42 which have shown differing processes of regulation. *S. meliloti* and *B. japonicum* both employ the *fixLJ-K* regulatory cascade. FixL directly senses O_2 concentration through a heme sensing domain and under low O_2 tension phosphorylates FixJ to then activate *fixK*. In *S. meliloti* FixJ also activates transcription of *nifA,* the low O_2 responsive *nifHDK* activator while in *B. japonicum nifA* is upregulated by the RegRS system and FixV in *Mesorhizobium loti* that both respond to as yet unknown signals. The FixK transcription factor then activates the transcription of *fixNOQP* and *fixGHIS* required for microaerobic respiration (Reviewed in Rutten and Poole, 2019). In other rhizobia such as *R. leguminosarum* and *R. etli* the hybrid pathway *hfixL-fxkR* is used, which then activates transcription of *fixK.* Also employed generally in species of rhizobia possessing *hfixL-fxrK* is *fnrN*, which like *fixL* directly senses O_2 and activates transcription of *fixNOQP* (Sullivan et al., 2013; Rutten and Poole, 2019).

While the genera above have been well studied in their regulatory networks, *Mesorhizobium* and specifically *Mesorhizobium ciceri* CC1192 has not had such attention. The vast majority of CC1192 symbiosis genes are encoded within the symbiosis integrative and conjugative element ICE*Mc*Sym[1192], although copies of *fixLJ and fixK* are present on the strains plasmid pMc1192, these have been found to be non-essential to nitrogen fixation (Hill et al., 2021). CC1192 has also been found to possess *fixV*, but unlike in *M. loti* R7A it is also not entirely essential. Deletion of *fixV* resulted in reduced, but not abolished, nitrogenase activity on a per nodule basis and reduced foliage dry weights (Figure 1) (Amiri, 2021). FixV, therefore, was found to be non-essential for N_2 fixation in CC1192 but likely plays a vital role alongside another regulator, potentially *nifA*, *fnrN or fixLJ*. Therefore, this study aims to characterise the phenotypes of *fnrN* and *nifA* mutant strains in *M. ciceri* CC1192, along with investigating *fixV* mutant phenotype in a plasmid cured background.

Figure 1. Symbiotic phenotype of fixV (MCC1138) and nifH (MCC139) mutants compared to wild-type CC1192 inoculated onto *Cicer arietinum* and grown for 42 days. Uninoculated controls were either nitrogen-fed (N+) or nitrogen-starved (N-).

To investigate the role of *fnrN* within *Mesorhizobium ciceri* CC1192, inactivation vectors were constructed ligating the suicide vector pJQ200SK, with roughly 1 Kb homologous regions upstream and downstream of the gene. A *nptII* cassette was ligated between these regions for marker replacement and fragments were assembled via Gibson cloning. Once the construct was confirmed via colony PCR and restriction mapping, it was mobilised into CC1192 via biparental conjugation. In order to cure pMc1192 a vector was designed firstly by ligating pSRK-Gm with the *sacB* gene and promoter for pJQ200SK for counter selection. Following this the *repABC* operon was cloned from pMESCI01 and ligated via Gibson assembly. This construct was then transformed into the *ΔfixV* phenotype previously generated. Currently both genotypes are being confirmed via Sanger sequencing. Following confirmation of genotype, *ΔfnrN,* pMc1192 cured *ΔfixV,* and *ΔnifA* genotypes will be assessed in glasshouse trials, where symbiotic phenotype of each will be assessed via ANOVA for the mean foliage dry weights and via acetylene reduction assays to measure nitrogenase activity.

Acknowledgements

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ALAN H. GIBSON MEMORIAL PRIZE

The Alan H. Gibson Memorial Prize will be awarded for the best student presentation, runner-up student presentation and best student poster at the 18th Australian Nitrogen Fixation Conference. This prize was initiated in 1999 at the 12th Australian Nitrogen Fixation Conference in Wagga Wagga, NSW, and has been awarded at subsequent Australian Nitrogen Fixation Conferences, SUNFix Symposia and other significant applied microbiology conferences. The prize is in memory of Alan's contribution and great enthusiasm for all things related to biological N_2 fixation.

Previous recipients

Year	Recipients	Conference
1999	Rosalind Deaker (University of Sydney) and Matthew Denton (University of Adelaide)	12th Australian Nitrogen Fixation Conference (Wagga Wagga, NSW)
2000	Wade Tozer (New Zealand) and Lucie Miché (France)	8th International Symposium on Nitrogen Fixation with Non-legumes (Sydney, NSW)
2001	Francine Perrine (Australian National University)	SUNFix Symposium 7 (Sydney, NSW)
2002	Jane Aiken (Western Sydney University)	SUNFix Symposium 8 (Sydney, NSW)
2002	Kemanthi Nandasena (Murdoch University) and Yvonne Cheng (Murdoch University)	13th Australian Nitrogen Fixation Conference (Adelaide, SA)
2003	Jodie Harris (University of Sydney)	SUNFix Symposium 9 (Sydney, NSW)
2004	Ryan Farquharson (Roseworthy, University of Adelaide)	SUNFix Symposium 10 (Sydney, NSW)
2005	Mick Rose (University of Sydney)	SUNFix Symposium 11 (Sydney, NSW)
2005	Bevan Weir (New Zealand) and Kavitha Somasundaram (Royal Melbourne Institute of Technology)	14th Australian Nitrogen Fixation Conference (Katoomba, NSW)
2006	Yvette Hill (Murdoch University)	SUNFix Symposium 12 (Sydney, NSW)
2006	Alison Bentley (University of Sydney) and Geraldine Mijajlovic (University of Sydney)	University of Sydney Faculty of Science Symposium (Sydney, NSW)
2007	Will Cuddy (University of NSW)	SUNFix Symposium 13 (Sydney, NSW)
2007	Michael Boyer (University of Lyon, France), Joel Pothier (University of Lyon, France), Roseline Remans (Katholieke Universiteit Leuven, Belgium) and Stijn Spaepen (Katholieke Universiteit Leuven, Belgium)	Azospirillum VII and related PGPR (Montpellier, France)
2007	Michelle McKechnie (University of Sydney) and Carlyn Kong (University of Sydney)	Microbes, Infection and Host Defence Showcase (Sydney, NSW)

2008	Macarena Gerding (Murdoch University)	SUNFix Symposium 14 (Sydney, NSW)
2009	Ganisan Krishnen (University of Sydney)	SUNFix Symposium 15 (Sydney, NSW)
2009	Sharon Fox (Murdoch University) and Julie Ardley (Murdoch University)	15th Australian Nitrogen Fixation Conference (Margaret River, WA)
2010	Akitomo Kawasaki (University of Sydney)	SUNFix Symposium 16 (Sydney, NSW)
2011	Meng-Han Lin (University of Queensland) and Nadiatul Akmal Mohd Radzman (Australian National University)	SUNFix Symposium 17 (Sydney, NSW)
2012	Victoria Clarke (University of Sydney)	16th Australian Nitrogen Fixation Conference, (North Head, NSW)
2014	April Hastwell (University of Queensland) and Mary Otieno (University of Sydney)	17th Australian Nitrogen Fixation Conference (Adelaide, SA)
2017	John Ramana (Lincoln University New Zealand) and Christopher Baldock (University of Sydney)	SUNFix Symposium 18 (Sydney, NSW)
2018	Talitha Rogers (Murdoch University) and Emma Bonello (Murdoch University)	SUNFix Symposium 19 (Sydney, NSW)
2022	Elaine Gough (University of Southern Queensland), Pinhui (Cindy) Wang (Australian National University) and Celine Mens (University of Queensland)	SUNFix Symposium 20 (Sydney, NSW)