**19th Australian Nitrogen Fixation Conference**

**Abstract Book**

**3rd – 5th October 2024**

**Brisbane, Australia**

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**ANFC 2024**





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| Thursday 3rd October 2024**ANFC 2024****3rd – 5th October 2024****Brisbane, Australia**International Conference on Legume Genetics and Genomics – Joint Session:Venue: Brisbane Convention & Exhibition Centre, South Brisbane |
| Time | Session | Location |
| 0730  | Registration open and information desk | Foyer |
| 0740  | Arrival tea and Coffee | Foyer |
| 0825  | Welcome Day 4 from Associate Professor Brett Ferguson |
| **Invited Talks**  | Boulevard Auditorium |
| 0830  | Theme: Beneficial symbiosis, Plenary Chair: Assoc Prof Brett Ferguson**Mechanisms of plant-microbe symbiosis and applications**Prof Ertao Wang |

**Plenary**

**Mechanisms of plant-microbe symbiosis and applications**

Ertao Wang1

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Nitrogen is the fundamental building block of key organic molecules in life processes, such as proteins, nucleic acids, and chlorophyll. It is essential for all living organisms. In the natural environment, nitrogen gas makes up 78% of the air. Plants, as primary producers in ecosystems, cannot directly utilize atmospheric nitrogen and rely on external nitrogen-containing compounds for their growth. To meet the demands of crop production, approximately 1-2% of global fossil energy is used for nitrogen fertilizer production. The excessive use of chemical fertilizers has led to severe environmental pollution.

In the symbiotic nitrogen fixation process, as seen in leguminous plants and rhizobia, nitrogen-fixing bacteria convert atmospheric nitrogen into forms of nitrogen compounds that are readily available for plant growth. This biological process reduces the dependence of leguminous plants on external nitrogen fertilizers. In my presentation, I will focus on mycorrhizal symbiosis and the nitrogen-fixing symbiosis between leguminous plants and microorganisms. I will discuss the nutrient exchange and signal recognition between plants and their symbiotic microorganisms, as well as the potential applications and challenges of plant-microbe symbioses in agricultural production.

Theme: **Symbiotic nitrogen fixation**

Location: Boulevard Auditorium

Chair: Assoc Prof Brett Ferguson and Dr April Hastwell

**Keynote**

**A genetic strategy to enhance nitrogen fixation in legumes**

Reid D1, Lin J2, Bjørk PK2, Kolte MV2, Stougaard S2, Andersen KR2,

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Plants adapt to fluctuating environmental conditions by adjusting their metabolism and gene expression to maintain fitness. In legumes, nitrogen homeostasis is maintained by balancing nitrogen acquired from soil resources with nitrogen fixation by symbiotic bacteria in root nodules. I will outline our approaches to identify novel regulators of nitrogen fixation in legumes. Unexpectedly, we found that zinc, an essential plant micronutrient, acts as an intracellular second messenger connecting environmental changes to transcription factor control of metabolic activity in root nodules. We identify a novel transcriptional regulator, FIXATION UNDER NITRATE (FUN), that acts as a sensor, with zinc controlling the transition between an inactive filamentous megastructure and an active master regulator. Lower zinc concentrations in the nodule, which occur in response to higher levels of soil nitrate, dissociates the filament and activates FUN. FUN then directly triggers breakdown of the nodule. The zinc-dependent filamentation mechanism thus establishes a concentration readout to adapt nodule function to the environmental nitrogen conditions. Our genetic and biochemical analysis thus identifies an unexpected regulatory mechanism and opens new possibilities for translation to the field.

**Keynote**

**The common bean (*Phaseolus vulgaris*) –*Rhizobium etli* N-fixing symbiosis: unraveling novel plant regulators through genetic/genomic approach**

**Hernández G1**, Reyero-Saavedra R1, Fuentes SI1, Leija A1, Jiménez-Nopala G1, San Vicente K1, Ramírez M1, Girard L1, Porch TG2, Peláez P1, Sánchez-Pérez M1.

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The symbiotic N2-fixation process (SNF) in the legume-rhizobia interaction, relevant for sustainable agriculture, is a complex process that is tightly regulated in both symbionts. Advances in legume genomics and genetics, mainly the characterization of symbiotic mutants, have been instrumental for the understanding of legume genes required for effective symbiosis with rhizobia [1].

Common bean (*Phaseolus vulgaris*) is the most important grain legume for human consumption in the world. The focus of our current research is to characterize or decipher relevant symbiotic genes in common bean using genetic approaches.

Only one common bean EMS symbiotic mutant has been genetically/molecularly characterized [2]. It is evident that isolation, characterization and mapping of symbiotic common bean mutants is required to expand the knowledge of the fine regulation (SNF) in this important crop. To this end, we have screened 1,692 M4 lines of an EMS-generated common bean mutant population derived from the BAT93 genotype [3]. After the population screening, we aim to characterize three stable non-nodulating *(nnod*) mutant lines, that appear to be recessive and monogenic. Microscopic analysis of inoculated mutants revealed that each line is altered in a different early step of rhizobial infection: effective root hair deformations, formation of infection chambers and infection thread formation and development [4]. Comparative whole genome sequence analysis was undertaken for the prediction of responsible mutated genes in each mutant. In this talk we will present advances aiming to decipher mutated genes as well as further genetical/molecular characterization of selected common bean mutants.

***References:***

*[1] Roy S. et al, Celebrating 20 Years of Genetic Discoveries in Legume Nodulation and Symbiotic Nitrogen Fixation. The Plant Cell. Vol.32, 2020, p. 15.*

*[2] Ferguson BJ. et al, The Soybean (Glycine max) Nodulation – Suppressive CLE peptide, GmRIC1, Functions Interspecifically in Common Bean (Phaseolus vulgaris) but Not in a Supernodulating Line Mutated in the Receptor PvNARK. Plant Biotechnology Journal. Vol. 12, 2014, p. 1085.*

*[3] Porch TG. et al, Generation of a Mutant Population for TILLING Common bean Genotype BAT93. Journal of American Horticultural Science. Vol. 134, 2009, p. 348.*

*[4] Reyero-Saavedra R, et al. Identification and Characterization of Common Bean (Phaseolus vulgaris) Non-Nodulating Mutants Altered in Rhizobial Infection. Plants. Vol. 12, 2023, p. 1310.*

**NF-Ys affect iron and nitrate homeostasis in *Medicago truncatula***

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Most legumes can interact with beneficial N-fixing bacteria called rhizobia. Shortly after contact with the rhizobia, components of the heterotrimeric Nuclear Factor Y (NF-Y) transcription factor complex, comprised of A, B and C subunits, are induced in the roots of the host. In *Medicago truncatula*, these subunits are encoded by multiple genes, 8 *NF-YA*s, 19 *NF-YB*s and 11 *NF-YC*s, of which *NF-YA1* has been implicated in rhizobial infection and nodule formation [1]. We investigated the role of *NF-YB7*, which is induced in *M. truncatula* root hairs in response to rhizobia and Nod factors [2]. We determined that NF-YB7 can interact with NF-YA1, and that loss of NF-YB7 reduced the formation of rhizobial infection threads. Contrary to expectation, *M. truncatula* roots overexpressing NF-YA1 and NF-YB7 (NFYox) showed suppressed nodule formation. Investigated of this phenomenon using RNAseq revealed that abscisic acid signaling is activated in NFYox roots. In addition, NFYox roots showed increased expression of *Ferritin* and deregulation of other iron related genes, and accumulated iron. Genes involved nitrate uptake and nitrate signaling were strongly repressed in NFYox roots, and comparison with ChIP-seq data revealed that amongst these, *NIN-like protein 1* and *Nitrate Transporter 1.1B* were direct targets. Co-expression of the ABA degrading enzyme CYP707A restored the ability of NFYox roots to nodulate. Our results suggest that ABA may serve as a negative feedback mechanism directly downstream of the NF-Ys during nodulation and reveals a potential role for NF-Ys in alleviating nitrate's negative effect on nodulation and in nodule iron homeostasis.

***References:***

*[1] Laloum T., et al. Two CCAAT-box-binding transcription factors redundantly regulate early steps of the legume-rhizobia endosymbiosis. Plant J. 2014, 79:757-68.*

*[2] Breakspear A., et al. The root hair "infectome" of Medicago truncatula uncovers changes in cell cycle genes and reveals a requirement for auxin signaling in rhizobial infection. Plant Cell. 2014 26:4680-701.*

**Nodule-specific PLAT domain (MtNPD1) - dependent host-strain compatibility in the *Medicago truncatula* – *Sinorhizobium* sp. symbiosis**

**Pislariu C I1,** Samara H1, Pradhan R1, Mehebub M S1, Pant C1

*1. Division of Biology, Texas Woman’s University, Denton, USA*

Symbiotic nitrogen fixation (SNF) is a complex process regulated by thousands of legume host and nitrogen fixing symbiont genes. Tobacco retrotransposon (*Tnt1*)-insertion mutagenesis has been instrumental in the discovery of new genetic controls of SNF in the model legume *Medicago truncatula*. A foward genetic screening identified the Nodule-specific Polycystin-1, Lipoxygenase, Alpha-Toxin (PLAT) Domain-encoding gene, *MtNPD1*, which is essential for root nodule development, survival of *Sinorhizobium* sp. inside infected cells, and efficient nitrogen fixation.

We previously reported that, in *npd1* nodules, *S. meliloti* Sm1021 fail to mature and undergo early senescence, while *S. meliloti* Rm41 differentiate and fix nitrogen, thus reversing the mutant phenotype into wild type. *MtNPD1* is predicted to play a role in facilitating or restricting effective nodulation in *M. truncatula* [1].

A systematic analysis of host strain compatibility using *Sinorhizobium* strains representing a range of secretion systems uncovered three *S. meliloti* strains that induce wild type-like nodules in *npd1*: Rm41, T073, and M10. In contrast, *S. meliloti* Sm1021 and *S. medicae* A321 and WSM419 induce deffective *npd1* nodules. A phenotype-informed pangenome analysis to identify relevant bacterial gene clusters will be discussed. Unique insights into paired transcriptional responses in the *MtNPD1*-mediated host-strain compatibility were gained by dual RNA-Seq. To facilitate the tracking of infections in single inoculations and in competition studies involving multiple strains, we developed a collection *Sinorhizobium* sp. strains constitutively expressing green-, red, cyan-, and yellow-fluorescent proteins. How reporters influence nodulation will also be discussed.

***References:***

*[1] Pislariu C.I. et al. (2019) The nodule-specific PLAT domain protein NPD1 is required for nitrogen-fixing symbiosis, Plant Physiology, vol. 180, no. 3, p. 1480.*

**Sugar signaling acts as a proxy for cytokinin signaling for de novo meristem formation during nodule organogenesis.**

**DasGupta M** 1, Molla F1, Kundu A1\*

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Symbiosis between plants and diazotrophs require formation of a *de novo* meristem for endocytic accommodation of symbionts, a process that is tightly regulated by plant hormones cytokinin and auxin. Cytokinin signaling through CRE1 receptor causes auxin accumulation by regulating its transport or biosynthesis to initiate cell division for nodule organogenesis. Accordingly CRE1 mutant (*cre1*) is unable to undertake symbiosis and our objective was to strategize and restore functional symbiosis in *cre1* for understanding the downstream events. Our findings are :- (i) Sucrose signalling can restore functional symbiosis in *cre1*. (ii) Sucrose signalling upregulated an auxin conjugate hydrolase *MtIAR33* that converts IAA-asp to IAA*.* Overexpression of *MtIAR33* could also restore symbiosis in *cre1* indicating deconjugation of auxin conjugates to be a potential pathway of auxin accumulation during nodule organogenesis. (iii) Sugar signaling significantly upregulated an auxin responsive homeobox transcription factor WOX5 well known for its role in meristem maintenance. While *AhWOX5* from *Arachis* having determinate meristem could completely restore symbiosis in *cre1, MtWOX5* from *Medicago* having indeterminate nodule meristem failed to do so. We could show that *MtWOX5* function as a repressor whereas *AhWOX5* acts as an activator and swapping a single amino acid is sufficient to functionally convert *MtWOX5* to *AhWOX5* and vice versa. Based on these evidences, we propose a model where we show CRE1-independent deconjugation of auxin to be a potential contributor to auxin accumulation and activation of NIN-WOX5 axis, a step forward toward having an integrated view of how organogenesis starts during root nodule symbiosis.

***References:***

1. Molla, F., Kundu, A. and DasGupta, M., 2023. Sucrose-induced auxin conjugate hydrolase restores symbiosis in a Medicago cytokinin perception mutant. *Plant Physiology*, *191*(4), pp.2447-2460.

2. Kundu, A., Molla, F. and DasGupta, M., 2019. Turanose mediated WOX5 expression rescues symbiosis in cytokinin perception mutant cre1. *bioRxiv*, p.830661.

**Root architecture is regulated by miR2111 and TML in response to soil Pi**

**Ferguson BJ1**, Zhang M1, Su H1, Grundy E1

*1. Integrative Legume Research Group, School of Agriculture and Food Sustainability, University of Queensland, St. Lucia, Brisbane, QLD, 4072, Australia*

Plants control their root system architecture in response to irregular and fluctuating nutrient availability in the rhizosphere. This includes localised morphological changes and drastic shifts in overall root system structure to enhance soil foraging and nutrient acquisition. Previously, we reported that enhanced miR2111 expression led to an increase in the density of emerged lateral roots (Zhang et al., 2021). Such a phenotype is often observed in Pi-deplete plants. This prompted us to hypothesise that the miR2111 and TML regulatory module that controls nodule organogenesis may have been hijacked and evolved from a pre-exiting regulatory mechanism that acts to alter root development in response to environmental factors, such as nutrient availability. We tested this hypothesis by functionally characterising miR2111 and TML in the context of Pi deprivation-induced root adaptive responses. Three *GmmiR2111* encoding genes were transcriptionally upregulated in leaves, but not roots, of Pi-starved plants, resulting in increased accumulation of mature miR2111 and a concomitant decrease in the transcript abundance of *GmTML1* homologous in roots. Overexpression of *GmTML1* encoding genes reduced lateral root density and root thickness. This was consistent with phenotypic alternations observed in Pi-starved root systems, which exhibited diminished root growth, enhanced root branching and increased root diameter compared with control plants. Collectively, these findings demonstrate that miR2111 and TML have a critical role in the systemic manipulation of root system architecture in response to Pi availability, and subsequently appear to have been co-opted into the nodulation control mechanism of legumes.

***References:***

Zhang MB, Su HN, Gresshoff PM, Ferguson BJ (2021) Shoot-derived miR2111 controls legume root and nodule development. Plant, Cell & Environment 44: 1627-1641.

**Genetics and genomics of symbiotic nitrogen fixation in legumes: past, present, and future**

**Michael Udvardi1**, Celine Mens1, Estelle Grundy1,2, Brett Ferguson1,2, Merrill Ryan3, Thomas Noble3, Eric Dinglasan1, Millicent Smith1, and Lee Hickey1.

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Legumes are a large and diverse family of plants that provide us with food, feed, fuel, and feedstocks for industry. They can use atmospheric di-nitrogen for growth, via symbiotic nitrogen fixation (SNF) with bacteria called rhizobia, making them key to sustainable agricultural systems. There are opportunities to increase SNF in legumes to help tackle critical challenges related to the overuse of fertiliser-N in agriculture. The last two decades have seen enormous progress in our understanding of the genetics of SNF, although this is yet to be leveraged to improve SNF in legumes. In principle, two main plant-based approaches exist to improve SNF, one involving genetic engineering and the other using existing natural variation for this complex trait. These approaches are not mutually exclusive and now is an opportune time to attempt to increase SNF in legumes via plant genetics and genomics. This presentation will briefly review current knowledge of SNF genetics before outlining potential pathways to SNF improvement in legumes. Importantly, there is a new national initiative in Australia to develop genetic resources and knowledge for predictive plant breeding to improve SNF in chickpea, lentil, field pea, fababean, lupin, and mungbean. An outline of this project will also be presented.

Theme: **Beneficial symbioses and microbiomes**

Location Boulevard Auditorium

Chair: Prof Ulrike Mathesius

**Keynote**

***Getting to the root of symbiotic root nodule development***

**Schiessl K.**1, Lee T.1,2, Orvosova M. 1,2, Oldroyd G.E.D2

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Legumes grow specialized root nodules to host beneficial nitrogen-fixing bacteria that provide the plant with ammonia in exchange for carbon. These symbiotic nodules are distinct from lateral roots in morphology and function as they comprise of cells that can accommodate nitrogen-fixing rhizobial bacteria intracellularly and provide favorable conditions for the biological nitrogen fixation process.

Nodules initiate from the inner tissue layers in response to the perception of rhizobial bacteria at the root surface via cytokinin-mediated upregulation of the nodulation-specific transcriptional regulator *NODULE INCEPTION (NIN)*. Our previous findings that the initiation of lateral roots and nodules converges at a common developmental program [1] led to the hypothesis that an additional nodule-specific program is required to determine nodule organ identity on top of the shared root-like initiation program.

Recently, we have shown that two members of the *LIGHT SENSITIVE SHORT HYPOCOTYL (LSH)* transcription factor family (*MtLSH1* and *MtLSH2*), predominantly known to define organ boundaries and meristem complexity in the shoot, function as regulators of nodule organ identity [2]. *MtLSH1*/2 are upregulated during early stages of nodule development in a cytokinin- and *NIN*-dependent manner and are expressed in dividing cells. Our loss of function analysis of *lsh1/2* demonstrated that these regulators are required for the development of functional nodule primordia that can support the intercellular cortical infection, the intracellular colonization, and nitrogen-fixation by the bacteria.

Furthermore, molecular functional analysis revealed that *LSH1/2* control components of the auxin-cytokinin cross talk and function upstream of and together with the previously identified nodule organ identity genes nuclear factor *Y*-*A1* (*NF-YA1)* and *NODULE ROOT1/2 (NOOT1/2)* to recruit a program with pleiotropic functions in the shoot to differentiate nodules from lateral roots and to determine nodule organ identity. The principal outcome of *LSH1/LSH2* function is the production of cells able to accommodate nitrogen-fixing bacteria, the unique nodule feature.

These findings provide a framework at molecular and cellular level to investigate how the coordinate recruitment of pre-existing organ development and identity programs can underpin the morphological and functional divergence between lateral roots and nodules, in parallel to a root initiation program.

**REFERENCES:**

[1] Schiessl et al., Curr Biol (2019).

[2] Lee, Orvosova et al., Curr Biol (2024).

**Genetic diversity and symbiotic effectiveness of *Mesorhizobium* and *Bradyrhizobium* strains nodulating selected annual grain legumes growing in Ethiopia**

**Tulu Degefu1**, Endalkachew Wolde-meskel2

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*Cicer arietinum L., Vigna unguiculata, Vigna radiata*, and *Arachis hypogaea* growing in Ethiopia are nodulated by a genetically diverse group of rhizobia where chickpea (C. arietinum L.) being nodulated by Mesorhizobium genus while the latter three host legumes are by Bradyrhizobium strains. A collection of 167 test strains originating from the root nodules of respective hosts was investigated using multilocus sequence analyses (MLSA) of core genes including 16S rRNA, *recA, glnII, gyrB, atpD* and *dnaK*. Sequence analysis of *nodA* and *nifH* genes along with tests for symbiotic effectiveness were undertaken. The MLSA grouped most test strains into several well-supported distinct positions. We found similar grouping for the *nodA* and *nifH* gene sequences of strains from Vigna unguiculata, Vigna radiata, and Arachis hypogaea where most of the test strains were clustered on one of a well-supported large branch that comprise Bradyrhizobium species from the tropics. Similarly, the *nodC* and *nifH* gene sequences of strains from C. arietinum showed a monophyletic origin and related to a clade representing three symbiovars. The symbiotic effectiveness of selected test strains revealed the presence of highly effective nitrogen fixers. It was concluded that Ethiopian soils are a hotspot for rhizobial diversity. This calls for further research to unravel as yet unknown rhizobia nodulating legumes growing in the country. In this respect, prospective research should also address the mechanisms of symbiotic specificity that could lead to high nitrogen fixation for legume production that could help to sustainably intensify cropping systems.

***References:***

*[1] Degefu T. et al, Genetic diversity and symbiotic effectiveness of Bradyrhizobium strains nodulating selected annual grain legumes growing in Ethiopia. International Journal of Systematic and Evolutionary Microbiology, 2018.*

*[2] Gunnabo A.H. et al, Phylogeography and Symbiotic Effectiveness of Rhizobia Nodulating Chickpea (Cicer arietinum L.) in Ethiopia, Microbial Ecology, 2021.*

**Uncovering The Role Of Gibberellin In Nodulation: Gibberellins Restrict Rhizobial Infection In The Epidermis And Promote Nodule Organogenesis In The Endodermis And Regulate Key Nodulation Genes**

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Legumes associate with rhizobia to access to atmospheric nitrogen in root nodules. This symbiosis requires the precise coordination of rhizobial infection in the epidermis and nodule formation in the inner root layers. Plant hormones play important roles in regulating these processes, with their spatial and temporal activation dictating nodulation progression. We employed a powerful approach of specific epidermal and endodermal complementation of severely gibberellin-deficient *na* pea mutants, coupled with a novel comparison strategy of RNA-seq gene expression data from tissue-specific complemented roots, which allowed the dissection of genes possibly regulating specific nodulation stages. Our findings reveal that gibberellins restrict epidermal infection, limiting bacterial entry and regulating infection thread progression and branching in the cortex. Moreover, we found that gibberellins are essential in the endodermis to promote nodule and lateral root development. In contrast, gibberellin presence in the epidermis does not affect nodule or root development. Careful comparison of RNA-seq data of epidermal and endodermal complemented and control *na* mutants allowed the identification of genes potentially involved in the regulation of infection and nodule organogenesis downstream of gibberellin. These include the transcription factors *NSP1* and *NSP2*, ethylene response factors *ERN1* and *ERN2* and flavone synthase *FNSII*, along with genes important for nodule organogenesis and symbiosome formation. Future studies could target selected candidate genes to determine their precise role in nodulation and their regulation by gibberellin. This knowledge will facilitate the integration of the roles of plant hormones in nodulation and root development processes and the gene expression networks behind these processes.

**A novel nucleotide-binding domain leucine-rich repeat receptor (NLR) involved in soybean nodulation**

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Legumes can exploit their relationship with nitrogen-fixing rhizobia to thrive in low nitrogen environments via the formation of symbiotic organs on their roots, termed nodules. The plant innate immune system has been shown to be a major determinant of nodule formation. For example, soybean nucleotide-binding domain leucine-rich repeat receptors (NLRs) have been shown to restrict symbiosis with certain strains of rhizobia upon recognition of specific rhizobia proteins. Here, we report the characterisation of a novel NLR of soybean which responds to Nod factors unlike previously reported NLRs. Interestingly, a promoter::*GUS* fusion revealed this gene is induced from early infection through to mature nodules. When overexpressed, the *NLR* reduces nodule number and size. When knocked-out via CRISPR genome editing, nodule diameter significantly increased but there was no change in nodule number. mRNA of the *NLR* undergoes alternative splicing to produce at least four different protein isoforms. NLRs in plant-pathogen interactions, often called *R*-genes, have recently been shown to form resistosomes capable of degrading nucleotides and facilitating cell death. Sequence analysis suggests the soybean NLR involved in nodulation has the necessary catalytic sites for this enzyme activity. New insight into this gene and our current understanding of the interplay of plant immunity in legume nodulation will be presented.

**Nodule organogenesis in Medicago truncatula requires local stage-specific auxin biosynthesis and transport**

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The importance of auxin in plant organ development including root nodule formation is well established. Using auxin reporter constructs the spatiotemporal auxin distribution pattern during nodule development has previously been illustrated. However, our understanding of how this pattern is built-up and maintained still remains elusive.

To this end, we studied how the auxin gradient visualized by DR5 expression patterns at different stages of nodule development in Medicago truncatula (Medicago), is correlated with the spatiotemporal expression patterns of known auxin biosynthesis and auxin transport genes. In addition, we record the MtPIN10-GFP expression pattern and polar positioning on the cell plasma membranes during nodule primordium development to investigate the auxin flux. RNA interference and the application of auxin synthesis blockers were used to demonstrate the relevance of biosynthesis and transport at the initial stages of the nodulation process.

Our results show that upon rhizobium inoculation, preceding the first mitotic activity, a specific set of MtYUCs and MtPINs as well as MtLAX2 are expressed in the pericycle contributing to the creation of an auxin maximum. Overall, we demonstrate that dynamic spatiotemporal expression of both, MtYUCs and MtPINs, result in specific auxin outputs in subsequent stages of nodule primordia and nodule meristem formation.

**Exploring potential benefits of biostimulant treatments in lupin cultivation**

**Loehrer M**1,2, Schaffrath U1,2

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Lupin is a valuable alternative protein source for both human food and animal feed. White- and narrow-leafed lupins are cultivated in Germany alongside traditional protein crops such as broad bean and pea and represent an attractive alternative to (GMO-) soybean from national- and international markets. Our project “P³roLucas” (Optimization of plant performance and products for lupin cascade use) aims at promoting and improving lupin cultivation in Germany and encompasses a wide range of research topics ranging from exploration of lupin alkaloids for industrial use to sequencing of the Andean lupin genome as a resource for future breeding programmes [1].

Central topic is the smart use of so called biostimulants as (partial) alternatives to traditional plant protection products which are increasingly being phased out under European and national regulatory policies. Therefore, we are exploring effects of commercially available biostimulants in narrow-leafed lupin (*Lupinus angustifolius*) on plant growth and protection against biotic- and abiotic stresses. Products based on *Bacillus* spec. were identified as the most promising candidates. We implemented a combined approach of seed treatments including the so-called seed-priming strategy, which is close to agricultural practice. In addition, the localisation of bacteria after seed treatments and their influence on plant development and induction of resistance to lupin anthracnose, were investigated. We present results from lab-scale experiments, currently being validated in field experiments and analyses at the metabolic- and transcriptomic level, using consolidated and newly generated genomic resources within this project.

***References:***

*[1] https://www.biosc.de/p3roLucas\_en*

**Non-rhizobial bacteria exhibit persistent colonization in the roots and nodules of chickpea cultivars across diverse environments**

**Zhou Y**1, Denton MD1

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Rhizobia are known for forming stable symbiotic relationships within the endosphere of legume hosts. However, the colonization of non-rhizobial bacteria in legume roots has been less explored. Firstly, we investigated the mechanisms for the microbiome establishment in the chickpea (*Cicer arietinum* L.) root across different cultivars and soils. Deterministic effects were more important than the stochastic processes in shaping the endophyte community structure for all the cultivars tested. The cultivar with higher symbiotic potential had greater secretion of benzoic acid (BA) and consistently colonised by 3 core ASV (Amplicon Sequence Variant) from *Burkholderia* clade in the root. Using a selective media, DNA identification, and colonisation testing, two of 98 isolated strains were confirmed as the culturable candidates of the core ASV. The two core strains were able to survival and grow in higher concentration of BA than the other strains, but had no plant growth promotion potential. In the second study, we analysed the nodule microbiome of four chickpea cultivars across five different growing environments spanning 1,400 km in Australia. Besides the symbiotic *Mesorhizobium*, we found two ASV from the *Burkholderia* clade and *Pseudomonas* sp. were persistently enriched in the nodules of all tested cultivars and environments. The culturable strains of these ASV were isolated, and demonstrated significant capability in producing Indole-3-Acetic Acid and enhancing chickpea nodulation and nitrogen fixation. The conserved colonisation of non-rhizobial endophytes is one target for future research developing beneficial strains to promote legume growth.

**Ethylene inhibits cell cycle progress in root hairs of *Lotus japonicus* infected by rhizobia**

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The symbiotic bacteria *Mesorhizobium loti* typically gains entry to its host *Lotus japonicus* by means of infection threads, tubular invaginations that serve as transcellular passageways through root hairs, that form through the physical remodelling of the cell wall and membrane. During rhizobial infection numerous cell division genes are induced, suggesting at least partial engagement of cell cycle related processes to provide an invasive impetus, involving reorganization of the cytoskeleton and enlargement of the nucleus [1, 2].

Here we show that ETHYLENE RESPONSE1 (ETR1) is involved in cell cycle regulation during infection thread establishment. We found that the ethylene insensitive mutant LjETR1-1 displays an enlarged, irregular shaped nucleus in infected root hairs, which is sometimes associated with blocked infections. We show that despite the nuclear enlargement that occurs in wild type infected cells, no *de novo* DNA synthesis takes place, while in LjETR-1 these cells enter S phase, highlighting a new role for ETR1 in the direction of cell cycle processes during symbiotic infection.

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**The NRT2.3 Nitrate Transporter Plays a Positive Role in Nodule Function Medicago truncatula**

**Fuyu L**.1,2, Murray J. D. 1,2,3

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Nitrate (NO3-) is the major inorganic form of nitrogen absorbed by plants and a key external environmental factor in regulating root nodule symbiosis and nitrogen fixation. As N2-fixation is relatively energy intensive, low nitrogen levels promote nodulation while high nitrogen inhibits nodulation and nitrogen fixation in legumes. Here, we show that *Medicago truncatula NRT2.3* encodes a plasma membrane localized high affinity nitrate transporter plays an essential role in balancing nitrate transport in roots and in nodules. Loss of *NRT2.3* in non-symbiotic conditions caused nitrate accumulation in roots while decreasing shoot nitrate content. Under symbiotic conditions, *nrt2.3* mutants formed fewer nodules, and the nodules that formed had decreased acetylene reduction activity. Moreover, *nrt2.3* nodules were greenish in color, showed symbiosome degradation, and had higher expression of the senescence marker gene *Cysteine Protease 6*. Finally, both high nitrate (5 mM KNO3) and low nitrite (0.5 mM KNO2) treatments promoted the formation of green nodules in *nrt2.3,* which was associated with higher levels of nitrate and nitrite in the mutants. From these findings we conclude that *MtNRT2.3* mediated nitrate transport in nodules is important for optimal nitrogen fixation.

**Tyrosine Sulphated Root Meristem Growth Factor Peptides Regulate Root and Nodule Development in Soybean**

**Yuhan Liu1**, Xitong Chu1,2, Brett Ferguson1, April Hastwell1

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Legumes form a beneficial symbiotic relationship with soil bacteria that can fix atmospheric nitrogen into a plant usable form, resulting in a reduced reliance on synthetic nitrogen fertilizer and the associated negative environmental issues. This process is known as nodulation and involves complex molecular signaling pathways to control the physiological changes that are required to initiate and maintain nodule organogenesis. We have identified novel tyrosine sulfated peptides, called Root Meristem Growth Factor (RGF) peptides, in one of the most widely produced legumes, soybean (*Glycine max*)1. They are homologous to Arabidopsis RGF/GLV/CLEL peptides that play essential roles in regulating meristematic activity and immune responses2. We have identified conserved orthologs in other agriculturally important legumes, including *Phaseolus vulgaris*, *Pisum sativum* and *Lotus japonicus.* Within the soybean gene family, we have functionally characterized five soybean RGF-peptide encoding genes using multidisciplinary molecular biology techniques. Our results identified that they are expressed in the root tip region where meristem is located and during different growing stages of lateral root growth and nodulation. They also demonstrate a systemic signal transduction pathway, which is not seen in Arabidopsis and necessary for proper root growth and nodule development. These findings enhance our understanding of legume signaling and symbiotic nitrogen fixation and benefit future legume crop development programs which will improve the impact agriculture has on the environment, human health, economy, and biodiversity.

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| Friday 4th October 2024Venue: Queensland Parliamentary House Annexe |
| Session | Location |
| Registration Open | Colonnade – Level 3 - Annexe |
| Welcome to Country: Greg Egert; & Welcome to ANFC: A/Prof Brett Ferguson |
| Session 1 |
| Theme: **Symbiotic Nitrogen Fixation – Microbes**, Chairs: Dr Jason Terpolilli & Kit BurnsLocation: Undumbi Room – Level 5 – Annexe |

**Keynote**

**What is a bacteroid?**

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A combination of RNAseq, Insertion sequencing, in frame mutagenesis, biochemical analysis and modelling of the developmental stages of bacteroids from determinate bean and indeterminate pea nodules were compared (1-5). This has enabled us to contrast the development of N2 fixing bacteroids in the different types of nodules. In the case of bean bacteroids, which retain the ability to regrow from bacteroids, we then examined the de-differentiation process by further Insertion sequencing and RNAseq. This revealed a clear hierarchy of adaption to the free-living bacterial state and loss of bacteroid properties. To put these developmental fates into context of whole plant physiology we have mapped the differences in nodule function in peas and beans, beginning with whole plant rates of N2-fixation down to individual bacteroids. To enable this acetylene reduction and 15N2 incorporation were determined at multiple scales in both plants. Serial SEM sectioning and single cell quantitative proteomics were then conducted to map this data to nodules and individual bacteroids. This has enabled us to develop model nodules for both determinate bean and indeterminate pea nodules, to compare the developmental fates, resource allocation and efficiency of N2 fixation in these systems.

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**Symbiotic Variations Among *Bradyrhizobium* Strains Inducing Nodulation in Soybean Plants Independent of Nod Factors**

Ratu STN1, Fukunaga S2, Okazaki S1,2

1 Institute of Global Innovation Research, Tokyo University of Agriculture and Technology, Tokyo, Japan

2 Graduate School of Agriculture, Tokyo University of Agriculture and Technology, Tokyo, Japan

Leguminous plants form a N2-fixing symbiosis with rhizobia. For a long time, the perception of rhizobial Nod Factors (NFs) via cognate host receptors (NFRs) was assumed to be a universal paradigm for inducing the signal transduction cascades required for infection and nodule organogenesis. Intriguingly, we discovered an alternative symbiotic interaction in soybean that is independent of NFs by utilizing a type III secretion system (T3SS). The T3SS commonly functions in pathogenic bacteria to deliver effector proteins (T3Es) into host plant cells.

We previously reported that *Bradyrhizobium elkanii* USDA61 employs the T3SS to initiate NF-independent nodulation. The T3 effector Bel2-5 of USDA61 has been identified as an indispensable factor for triggering NF-independent nodulation in soybean. However, the presence of Bel2-5 causes strong nodulation restriction of USDA61 in soybean carrying the *Rj4* allele by activating plant immune responses.

In current study, we discovered other *Bradyrhizobium* strains capable of inducing nodulation independently of the NFs signal. Intriguingly, these NF-independent nodulation strains exhibit variations distinct from USDA61, including: (a) symbiotic efficiency with *Rj4* soybean, and (b) the presence of divergent form or absence of the Bel2-5 effector in their genomes. Together, these results provide insights into the mechanisms by which diverse *Bradyrhizobium* strains induce NF-independent nodulation in soybean, including the mechanisms, prevalence, and significance of the divergent forms or absence of the Bel2-5 effector used by different *Bradyrhizobium* strains."

**Utilising nodulation genes to predict host range of *Mesorhizobium ciceri* strains WSM1497 and WSM1284**

Robbins G1, O’Hara G1, Terpolilli J1

1 Legume Rhizobium Sciences, Murdoch University, Perth, WA

*Biserrula pelecinus*, an herbaceous annual legume, was introduced into Australia from the Mediterranean basin in the 1990s to provide a hard-seeded drought-tolerant pasture.1 Australian soils lack native rhizobia capable of nodulating and fixing N2 with *B. pelecinus*, leading to the introduction of inoculant strain *Mesorhizobium ciceri* WSM1497.2 Strains effective on *B. pelecinus* differ in their host range. While WSM1497 has a narrow host range only effectively nodulating *B. pelecinus*. In contrast, *M. ciceri* WSM1284has a broad host range effectively fixing N2 with *B. pelecinus* and *Lotus ornithopodioides*,and ineffectively nodulating other important pastures.3 Understanding host specificity differences among these *Mesorhizobium* strains could improve inoculation selection practices in Australia.

WSM1497 and WSM1284 genomes are highly similar yet, the strains differ in nodulation genes indicating the strains may synthesise different Nod factor signalling molecules. WSM1284 encodes 21 *nod* genes, while WSM1497 has 14. Six of the additional WSM1284 genes code for fucose biosynthesis (*noeL*, *nolK*, *noeJ*, *noeK*), transfer (*nodZ*) and acetylation (*nolL*). Both strains harbour multiple copies of divergent *nodD* indicating these NodDs may respond to different host flavonoids. Eighteen WSM1284 site-directed mutants targeting putative Nod factor transcriptional regulators (encoded by *nodD*) and biosynthesis genes (encoded by *nodA*, *nodZ* and *nolL*) have been evaluated *in planta* on *B. pelecinus, L. ornithopodioides, Ornithopus sativus* and *Leucaena leucocephala.* This study will increase our understanding of regulation of host range to improve the current inoculant selection process.

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**Transcriptomic analysis of *Cicer arietinum* endosymbiont *M. ciceri* CC1192 in free-living and symbiotic conditions**

Kohlmeier MG1, Hill YJ1, O’Hara GW1, Terpolilli JJ1

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*Cicer arietinum* (chickpea) is an annual legume that is grown in more than 600,000 hectares throughout Australia and is recommended to be inoculated with *Mesorhizobium ciceri* CC1192 [1]. This strain has a sequenced genome which consists of a 6,943,628 bp chromosome and a single plasmid, pMc1192, that is 648,231 bp in size. Like other strains of *Mesorhizobium*, CC1192 houses it symbiosis genes (*nod*/*nif*/*fix*) on a chromosomal, 419 kb, Integrative and Conjugative Element (ICE) [2]. This ICE has been shown to transfer to nonsymbiotic soil bacteria, resulting in the generation of novel symbionts with varying levels of symbiotic performance [3]. A plasmid cured derivative, MCC70, was generated to elucidate the role of pMc1192 and was shown to be symbiotically effective [3], however the plasmid may yet still have some larger metabolic and/or symbiotic role.

Therefore, to better understand gene expression in these strains, total RNA was harvested from both *M. ciceri* CC1192 and MCC70 in free-living and symbiotic conditions. The samples were subject to RNA sequencing to determine valuable gene expression data in these varied lifestyle conditions. The data suggests the utilization of the Entner-Doudoroff pathway for glycolysis and Embden-Meyerhof-Parnas pathway for gluconeogenesis. Symbiosis genes and Tricarboxylic acid cycle genes are upregulated during symbiosis. Additionally, subtle changes in symbiosis gene expression in MCC70 suggest a regulatory role for pMc1192 during symbiosis.

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*[1] Farquharson EA et al. Inoculating Legumes: Practice and Science: Grains Research and Development Corporation; 2022.*

*[2] Haskett T et al. Complete genome sequence of* Mesorhizobium ciceri *strain CC1192, an efficient nitrogen-fixing microsymbiont of* Cicer arietinum*. Genome Announc 2016;4(3):e00516-00516.*

*[3] Hill Y et al. Evolution of diverse effective N2-fixing microsymbionts of* Cicer arietinum *following horizontal transfer of the* Mesorhizobium ciceri *CC1192 symbiosis integrative and conjugative element. Appl Environ Microbiol 2021;87(5):e02558-02520.*

Characterisation and effectiveness of potential inoculants for the novel pasture legume, *Scorpiurus muricatus*

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*1 Legume Rhizobium Sciences, Murdoch University, Perth, Australia*

*2 Department of Primary Industries and Regional Development, Perth, Australia*

Agricultural productivity in the medium-to-low rainfall areas of southern Australia is constrained by a lack of suitable annual pasture legumes. *Scorpiurus muricatus* is an annual legume from the Mediterranean basin with potential to fill this niche. A highly effective inoculant strain is essential to support the introduction of *S. muricatus*, but little is known about the diversity and effectiveness of *S. muricatus*-nodulating bacteria. Therefore, this study genotypically and symbiotically characterised *S. muricatus* microsymbionts, to identify a potential inoculant strain.

Genomic analysis of 39 *S. muricatus*-nodulating strains from Australia (5), Croatia (2), Israel (11), Morocco (4) and Sardinia (17), identified 36 strains as *Mesorhizobium*, andthree strains of *Bradyrhizobium*. Symbiotic characterisation showed that 35 of the 36 *Mesorhizobium* strains fixed N2 with *S. muricatus*, while all three *Bradyrhizobium* strains ineffectively nodulated this host.

Field performance of the four most effective *Mesorhizobium* strains (WSM1343, WSM1386, WSM4821 and WSM4842) was evaluated at two field sites in WA. Uninoculated controls at both sites were nodulated, forming small, pale pink finger-like nodules which were occupied by *Bradyrhizobium* spp. In contrast, inoculated plants formed large pink coralloid nodules, occupied by inoculant *Mesorhizobium* strains. WSM1386 was the best performing strain at both sites, as determined by percentage of nodulated plants, nodule weight, shoot N-content and N2 fixation (as measured by 15N natural abundance).

This indicates that *S. muricatus* responds to inoculation in the field, with WSM1386 a potential inoculant strain for this new pasture legume.

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| Session 2 |
| Theme: **Symbiotic Nitrogen Fixation – Microbes**, Chairs: Dr Graham O’ Hara & Jordan DavisLocation: Undumbi Room – Level 5 – Annexe |

**Keynote**

**Evolution and effectiveness of symbiotic N2 fixation in rhizobia**

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Rhizobia have comparatively large and complex genomes, consisting of a chromosome with additional plasmids and/or chromids of varying sizes. The location of symbiosis genes (i.e. *nod*, *nif* and *fix*) in rhizobial genomes is genus-specific, with some genera (e.g. *Sinorhizobium* and *Rhizobium*) encoding these genes on plasmids, while others (e.g. *Mesorhizobium* and *Bradyrhizobium*) encode them chromosomally. In *Mesorhizobium* spp., symbiosis genes are chromosomally encoded on Integrative and Conjugative Elements (ICEs), which can excise from the donor chromosome, conjugally transferring and integrating into a recipient chromosome at specific conserved sequences. Symbiosis ICEs can be monopartite or tripartite in structure and share related ICE transfer and regulation genes, suggesting they evolved from a common ancestral ICE1,2. Symbiosis ICE conjugal transfer, integration and excision is regulated such that ICEs are not readily lost from host genomes and can be difficult to cure from strains *in vitro*. In soil, symbiosis ICEs can transfer from inoculant *Mesorhizobium*, resulting in the evolution of new legume microsymbionts1,3,4. ICE recipients appear to be non-symbiotic soil *Mesorhizobium* spp., which presumably form part of the normal microbial community2. Some strains that receive symbiosis ICEs may not fix N2 efficiently, even though the ICE originated from a highly effective donor strain2-4. These strains may outcompete inoculant rhizobia for nodulation of the target legume, reducing the benefits of inoculation. Managing the detrimental impact of suboptimally effective strains on symbiotic N2 fixation requires the development of culture-independent methods to distinguish between inoculant and evolved nodule occupants in field nodulated legumes.

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Symbiotic performance of chickpea mesorhizobia with diverse ICESyms

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Transfer of symbiotic integrative conjugative elements (ICESyms) between mesorhizobia gives rise to genotypes with variable symbiotic performance in chickpea (*Cicer arietnum*). ICESyms in chickpea mesorhizobia may be tripartite or monopartite [1], however, only mesorhizobia contining the monopartite ICE*Mc*Sym1192 have been recovered from chickpea nodules in Australia originating from commercial inoculation with *Mesorhizobium ciceri* CC1192 [2],[3]. Variation in the core genome has been identified as a potential driver of differential symbiotic performance in rhizobia [3],[4]. Sequencing of complete genomes revealed five genetically distinct monopartite and tripartite ICESyms in chickpea nodulating mesorhizobia in the SUNFix culture collection. Strains isolated from nodules of Australian grown chickpea contained ICE*Mc*Sym1192. Variable ICESyms were identified in strains originally from international collections ICRISAT and ICARDA. Symbiotic performance of mesorhizobia with different ICESyms was variable across a diverse range of chickpea and wild *Cicer* (*Cicer echinospermum* and *Cicer reticulatum*) accessions. While performance varied with host genotype, three strains with monopartite ICESyms performed signifcantly better on average than four other strains across seven chickpea genotypes. Two strains carrying genetically distinct tripartite islands were generally poorer performing across host genotypes including wild *Cicer*. Only one strain was able to consistently nodulate *C. echinospermum* accessions. Phylogenetic analysis of core and accessory genes from this strain indicate close alignment of core genes with a nodule isolate from *C. echinspermum* [1]. The results further support a relationship between rhizobial core genes and host interactions as well as a potential risk of introducing mesorhizobia with genetically diverse ICESyms to Australian chickpea growing regions.

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*[1] Greenlon A. et al, Proc Natl Acad Sci USA, vol. 116, no. 30, 2019, pp. 15200–15209*

*[2] Hill Y. et al, Appl Environ Microbiol, vol. 87, no. 5, 2021, pp. 1–16*

*[3] Hill Y. et al, Plant Soil, 2024*

*[4] Haskett TL et al., Proc Natl Acad Sci U S A, vol. 113, no. 43, 2016, pp. 12268–12273*

**The evolution of chickpea nodulating *Mesorhizobium* through ICE*Mc*Sym1192 transfer in Australia: Are there alternative ICE’s?**

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*Mesorhizobium ciceri* symbiovar (sv.) *ciceri* CC1192 was introduced into Australia from Israel in the 1970’s as a highly effective strain for *Cicer arietinum*. Since this time, CC1192has been the only commercial inoculant for this grain legume [1]. In spite of this and an apparent lack of pre-existing compatible rhizobia for this grain legume in Australian soils, several studies have now reported the isolation of newly evolved rhizobia, genetically distinct to CC1192 that are capable of nodulating *C. arietinum* [2]. These strains have acquired the mobile 419-kb *M. ciceri* CC1192 symbiosis ICE (ICE*Mc*Sym1192) by environmental transfer from the inoculant strain [2, 3]. Some of these newly evolved rhizobia are as effective as CC1192 at fixing N2 with *C. arietinum*, but others are suboptimally efficient. While the genetic basis of these different phenotypes is unclear, symbiosis genes from diverse and geographically dispersed *Cicer-*nodulating *Mesorhizobium* spp. are generally monophyletic. This suggests that the cause of suboptimal effectiveness is unlikely to be due to classical symbiosis genes encoded on the ICE. However, ICE’s carry numerous non-symbiosis cargo genes with unknown functions. Bioinformatic analysis of available genome sequences of *C. arietinum* nodulating rhizobia symbiosis ICEs shows substantial genetic recombination between ICE cargo genes [4]. It is therefore possible that cargo genes have an important symbiotic role and that differences in ICE cargo gene complement and function may influence N2 fixation effectiveness. Exploring these possibilities may highlight important genomic traits that could be exploited for inoculant development.

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**A preliminary snapshot of diversity within mungbean nodulating rhizobia in the northern growing regions of Australia**

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Green (*Vigna radiata*) and black (*V. mungo*) mungbeanare high value, short duration legumes well suited to the tropical conditions of the northern agricultural regions of Australia, where the vast majority of the country’s 100,000 tonne/year production occurs [1]. Mungbean is known to form symbioses with multiple genera of rhizobia. However, *Bradyrhizobium* species are most frequently reported as nodule symbionts [2-4]. The current Australian commercial inoculant for mungbean is *Bradyrhizobium* sp. CB1015, which originated from India [5]. A previous study conducted in the Burdekin growing region found that at one site that had been inoculated with CB1015 six years previously, mungbean was not commonly nodulated by CB1015, with more than 90% of isolates representing other *Bradyrhizobium* genotypes [4].

To gain a snapshot of diversity among mungbean nodulating *Bradyrhizobium*, strains were isolated from nodules of mungbean cultivated in Queensland (12 sites), and the Ord River growing region of northern WA (2 sites). These strains were first genotyped by PCR fingerprinting, before whole genome sequencing was performed. Initial results from 11 strains, based on whole genome phylogeny, dDDH, and ANIb values showed the strains grouped into four species, with the largest number of strains clustering with *B. yuanmingense*. However, *nod* gene phylogeny showed all 11 strains grouped closely to *B. yuanmingense*. The remaining three groups represent potentially novel *Bradyrhizobium* species, with this number expected to expand when whole genome sequencing is completed on remaining isolates from Queensland and the Ord River isolates.

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**Some wild bradyrhizobia promote mungbean growth or fix nitrogen as well as the commercial strain under abiotic stress.**

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Mungbeans are often poor nitrogen fixers compared with other legumes. We investigated whether some wild bradyrhizobia strains may be equal or superior to the commercial inoculum, CB1015, in promoting mungbean growth or nitrogen fixation under a series of abiotic stresses.

Three small experiments were conducted under glasshouse or growth cabinet conditions, comparing the performance of mungbean plants when inoculated with CB1015 and wild bradyrhizobia collected from north Queensland. Plants were exposed to neutral and acid soil conditions, high and low air temperatures, and two water deficit regimes.

Under both acid and neutral soil conditions, thirteen of the fifteen strains evaluated promoted growth at least as well as CB1015. Two wild strains significantly outperformed CB1015 under neutral conditions. Though differences were found under acid conditions, none was significantly superior to CB1015.

Under both high and low temperature conditions, most of the eleven wild strains evaluated promoted mungbean growth at least as well as CB1015. Some wild bradyrhizobia significantly outperformed CB1015 for fixed nitrogen in shoots per pot. Some performed well at the high temperature (35°C), some at the low temperature (21°C), and some at all temperatures tested (21°C, 28°C, 35°C).

At least seven of the fourteen wild bradyrhizobia evaluated performed as well as CB1015 in well-watered conditions and both drought treatments.

Combined, these findings suggest that gains could be made in mungbean performance through use of better-adapted bradyrhizobia and that there may be significant untapped resources of wild bradyrhizobia that could be exploited to improve performance of mungbeans in northern Australia.

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| Session 3 |
| Theme: **Symbiotic Nitrogen Fixation – Plants**, Chair: Dr April Hastwell & Alexandria MattinsonLocation: Undumbi Room – Level 5 – Annexe |

**Keynote**

**Single-cell sequencing on soybean nodules identifies genes facilitating rhizobium infection**

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Legumes form symbiosis with rhizobium leading to the development of nitrogen-fixing nodules. Nodule development begins with the differentiation of cortex of infected root and involves interplays of both nodules and rhizobia. However, our understanding of cellular heterogeneity and developmental lineage of nodule is still limited. By integrating single-nucleus and spatial transcriptomics, we established a cell atlas of soybean nodules and roots. In central infected zones of nodules, we found that uninfected cells specialize into functionally distinct subgroups during nodule development, and revealed a transitional subtype of infected cells with enriched nodulation-related genes. Further functional analysis revealed the important role of cell-type-specific genes in regulating nodule development and nitrogen fixation.

***References:***

*[1] Liu Z. et al, Integrated single-nucleus and spatial transcriptomics captures transitional states in soybean nodule maturation, Nat Plants. 2023, 9(4):515-524.*

**Making the most of single cell data in legume-rhizobia symbiosis**

Frank M1, Fechete LI1, Tedeschi F1, Nadzieja M1, Nørgaard MMM1, Montiel J1, Andersen KR1, Schierup MH1, Reid D2, Andersen SU1

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Single cell expression data is now available for many plant species, including both model and crop legumes. This has provided opportunities to explore in detail a number of symbiotic events including the transcriptional dynamics in the few root hair cells that are infected by rhizobia.

We used this data to identify high-confidence nodulation gene candidates based on their specific expression in these root hair populations, pinpointing genes stably associated with infection across genotypes and time points. One of these genes, which we name SYMRKL1, encodes a protein with an ectodomain predicted to be nearly identical to that of SYMRK and is required for normal infection thread formation.

While this data is extremely powerful for hypothesis generation, the data is complex and specialised analysis techniques are required. I will discuss how to explore this data, giving examples from our datasets in how to identify cells of interest to support hypothesis generation in diverse projects focused on legume biology.

**References*:***

*[1] Frank, M., Fechete, L.I., Tedeschi, F. et al. Single-cell analysis identifies genes facilitating rhizobium infection in Lotus japonicus. Nat Commun 14, 7171 (2023). https://doi.org/10.1038/s41467-023-42911-1*

**A symbiotic flavonoid map of *Medicago***

van Noorden G1, Meade A1, Wang C1, Rae A1, Li Y1, Boyd J1, Martin S1, Mathesius U1

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Flavonoids are essential signals for nitrogen fixing symbioses in legumes, and have long been known to activate rhizobial nodulation genes, but they also have other functions as signals in the rhizosphere [1]. In the *Medicago-Sinorhizobium* symbiosis, flavonoids have functions in nodule initiation [2] that are unique to the development of nodules, in contrast to other root organs such as lateral roots and root galls [3]. The action of flavonoids in nodule development requires cytokinin signaling and appears to be linked to a legume-specific response in the root cortex, via action on auxin transport [4]. However, the identity of the flavonoids for most functions remains unknown.

Here, we examined the spatial and temporal changes in flavonoid metabolites during the early stages of nodulation in *M. truncatula*. We constructed a map of flavonoid accumulation in infected root hairs, root shaft, root tip and root exudate, and identified cell type-specific metabolite changes. Some of these agree with the known function of flavonoids that act as *Nod* gene inducers in *Sinorhizobium*, but several others that are not active as *Nod* gene regulators were identified.

We then surveyed the induction of flavonoids across different accessions of *M. truncatula* as well as in different *Medicago* species to assess the specificity of the responses. A comparison of flavonoid exudates from roots and seeds was made to establish likely concentration ranges of different flavonoids in the rhizosphere. Concentrations of several flavonoids correlated significantly with improved nitrogen fixation, suggesting that root (exudate) flavonoid composition could be an important breeding target.

**References*:***

*[1] Hassan, S. and Mathesius, U. (2012) The role of flavonoids in root-rhizosphere signaling - opportunities and challenges for improving plant-microbe interactions. Journal of Experimental Botany 63: 3429-3444*

*[2] Wasson (2006)**Silencing the flavonoid pathway in Medicago truncatula inhibits root nodule formation and prevents auxin transport regulation by rhizobia. Plant Cell 18, 1617-1629*

*[3] Wasson et al. (2009) Differing requirements for flavonoids during the formation of lateral roots, nodules and root knot nematode galls in Medicago truncatula. New Phytologist 183: 167–179*

*[4] Ng et al. (2015) Flavonoids and auxin transport inhibitors rescue symbiotic nodulation in the Medicago truncatula cytokinin perception mutant cre1. Plant Cell 27: 2210-2226*

**Silicon mitigates drought stress and supports carbon-nitrogen dynamics and biological nitrogen fixation in lentil plants.**

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Although the role of silicon in improving drought stress tolerance in lentils is well-established1-2, its involvement in root nodule metabolism with biological nitrogen fixation (BNF) is still unknown. This study aimed to investigate carbon-nitrogen metabolism in the nodules of drought-stressed lentils, with Si supplied during the onset of the flowering stage, a critical phase for nodule development and drought sensitivity.

The experimental design was completely randomised with drought treatments in four replications. Lentil genotypes were exposed to moderate (40-45% field capacity) and severe (20-25% field capacity) drought in a growth chamber, with and without silicon treatment. Various physiological, biochemical, and BNF efficiency-related traits were studied. Data were analysed using MATLAB, two-way ANOVA, Tukey test, and principal component analysis.

Silicon supplementation increased chlorophyll, photosynthetic rate, and chlorophyll fluorescence in drought-stressed plants and improved carbon-nitrogen dynamics with a total variance of 85.74% (P≤0.05). Silicon led to an increase in the activities of enzymes like nitrogenase, nitrite reductase, and nitrate reductase in nodules of drought-stressed plants while reducing the activities of sucrose synthase and invertase compared to control (p≤0.001). Furthermore, silicon facilitated ammonia assimilation in nodules under drought by significantly increasing the activities of glutamate synthetase, glutamate synthase, and glutamate dehydrogenase. A positive correlation was observed between the number of nodules, BNF efficiency, photosynthetic rate, final biomass, and harvest index (r=0.91-0.98). These findings strongly support the potential of silicon supplementation in lentils to enhance BNF during drought and validate the importance of maintaining a balance of enzymes and metabolites associated with carbon-nitrogen metabolism.

**References*:***

*[1] Biju, S. et al., Regulatory role of silicon on photosynthesis, gas-exchange and yield related traits of drought-stressed lentil plants, Silicon, vol. 15, no. 14, 2023a, 5981-5996.*

*[2] Biju, S. et al., Novel insights into the mechanism (s) of silicon-induced drought stress tolerance in lentil plants revealed by RNA sequencing analysis. BMC Plant Biol, vol. 23, no. 1, 2023b, 498.*

**Roots to seeds: Discovering and utilizing nodulation genes for high-yielding soybean**

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2 College of Resources and Environment, Fujian Agriculture and Forestry University, Fuzhou, China

Symbiotic nitrogen fixation (SNF) in legumes is beneficial to the environment, yet it requires a substantial carbon investment from the host plants. In past decades, a lack of success in yield improvement led to limited interest in breeding for increased SNF in legume crops. We have previously developed high-efficiency soybean genome editing system. and generated a collection of nodulation mutants in soybean 1. We found that soybean genome editing mutants with a moderate increase in nodules, rather than supernodulation mutants, has balanced nitrogen and carbon allocation, and is accompanied by enhanced plant growth. We performed multi-year, multi-site field trials, and observed a 10–31% increase in grain yield and significantly higher protein contents of the mutants with optimized nodulation over their elite progenitor control 2. Thus, bioengineering for a new equilibrium of symbiotic nitrogen fixation and carbon allocation improves soybean yield and protein content 2. So far, a significant bottleneck for optimizing SNF is the inhibition effects caused by the heavy use of N-rich fertilizers. We are now comprehensively characterizing the N-orchestrated regulatory network in soybean nodules, and have identified key regulators such as SNAP1/2/3/4 3, NIGT2a/2b transcription factors. The characterization of regulatory networks of would contribute to molecular breeding toward optimal nodulation and SNF for higher yield and quality in soybean.

**Reference:**

*[1] Bai, M. et al.. (2020 ) Plant biotechnology journal* ***18****, 721-731*

*[2] Zhong, X. et al. (2024) Nature plants* ***10****, 736-742*

*[3] Wang, X. et al. (2023) Nature Communications* ***14****, 4711*

**Non-Nodulating Mutants to Quantify Nitrogen-Fixation of Legume Crops**

Weston-Olliver G1, Hastwell A1, Williams B2, Zhang M 1,3, Ferguson B1

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*3 Institute of Molecular Biology, The University of Queensland, St Lucia QLD 4072, Australia*

Legume plants recognise compatible rhizobia partners through the perception of signal molecules, called Nod factors. These molecules are recognised by cross membrane LysM receptor kinases called Nod factor receptors that are located on the host plants roots. The receptors form a complex comprised of NFR1 and NFR5. Perception of compatible Nod Factor signals initiates infection thread formation and nodule development, with knock-out mutations in either receptor resulting in a non-nodulation phenotype.
Non-nodulating legumes can be useful tools in crop breeding programs. They are only able to acquire nitrogen from the soil, compared with commercial varieties that source nitrogen from both soil and through nitrogen-fixation. These mutants can be used to measure the extent rhizobia nitrogen-fixation has on the nitrogen content of varieties that can form nodules.
We have identified NFR5 orthologs in mung bean (Vigna radiata) and cowpea (Vigna unguiculata). Using CRISPR/Cas9 and Agrobacterium tumefaciens mediated stable transformation, we are now generating knockout mutants in both species that can be used in future trials as a baseline for quantifying the extent nitrogen-fixation contributes to nodulating plants. Updates on current progress will be presented.

**Regulatory network of nodule senescence pathway of the transcription factor FUN identified using DAP-seq**

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The process of fixing nitrogen from the atmosphere is an energy intensive process and legumes will therefore preferentially utilise available nitrogen in the soil before fixing from the atmosphere. Fine-tuning and enhancing the nitrogen fixation process will prove crucial to increasing crop yield (for both legumes and non-legumes) and for sustainable agriculture to feed an ever-growing population.

Recently, a key regulator of the nodule senescence process was identified as the TGA transcription factor FUN (fixation under nitrogen) [1]. It was identified by the application of nitrate treatments which are normally restrictive conditions for the nitrogen fixation process [1]. This project aims to investigate the gene regulatory network of FUN to further understand the biological processes and molecular mechanisms of nitrate-induced nodule senescence. DNA binding domains from *Vicia faba* and *Vigna unguiculata* FUN were expressed recombinantly and subjected to DAP-seq (DNA-affinity purification sequencing) [2] to identify genes under the regulatory control of FUN and provide a greater understanding of the molecular mechanisms of nodule senescence. By delineating the regulatory network of the nodule senescence process, it is thought that this process will identify molecular targets which could be manipulated to enhance nitrogen fixation and senescence processes.

**References:**

*[1] Lin et al. (2024).* Nature***631****: 164-169. doi:* 10.1038/s41586-024-07607-6

*[2] Bartlett et al. (2017).* Nat. Protoc.***12****: 1659-1672. doi:* 10.1038/nprot.2017.055

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| Session 4 |
| Theme: **Symbiotic Nitrogen Fixation- plants**, Chairs: Prof Ulrike Mathesius & Grace Weston-OlliverLocation: Undumbi Room – Level 5 – Annexe |

**Keynote**

**Root Meristem Growth Factor (RGF) peptides regulate soybean legume nodule development**

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2 College of Life Science and Technology, Huazhong Agricultural University, Wuhan 430070, China

The development of mature nitrogen fixing nodules requires coordinated molecular and development responses. Such molecular resources provide us with avenues to identify and modulate nodulation to benefit yields and conserve natural resources. However, many genes, proteins and other molecules remain unknown. New molecules that regulate these processes can be identified using systematic phylogenetics which can isolate novel genes and proteins that are specific to legumes. In particular, this is a useful strategy for small signalling peptide families.

Root meristem Growth Factor (RGF) peptides are a signalling peptide family that are well understood in the context of roots [1] and more recently, in the development of nodules [2]. We have identified the family of RGF peptide-encoding genes in soybean and identified legume specific genes using phylogenetics. Functional characterisation of these specific genes revealed they are induced upon rhizobial inoculation and have potential roles in root and nodule development, with plants overexpressing RGF peptides having reducing nodule number and reduced nodule maturation. Furthermore, one of the examined peptides may be involved in a novel systemic pathway to control legume development. These results will have implications for further refining legume genetics to improve symbiotic efficacy.

**References*:***

*[1] Hastwell A H et al, The parallel narrative of RGF/GLV/CLEL peptide signalling, TIPS. Accepted July 2024*

*[2] Roy S et al, The peptide GOLVEN10 alters root development and noduletaxis in Medicago truncatula. Plant J. 2024 118(3):607-625*

**GRDC updates and future directions**

Martinez, C1

*1Grains Research and Development Corporation*

**Roles of UmamiT amino acid Transporters in nodule function**

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*2 Syngenta, North Carolina, USA.*

*3 Universidad Pública de Navarra 31006 Pamplona, Spain*

The establishment and maintenance of a symbiotic interaction between rhizobia and legumes requires a coordinated exchange of resources during the early-infection and nitrogen-fixation stages of root nodulation. We investigated the role of members of the Usually Multiple Acids Move In and out Transporter (UmamiT) family in enabling resource exchange in the Medicago-Sinorhizobium symbiosis. We identified eight UmamiT family members that show nodule-specific expression and named them UmamiT Nodulins (UTNs). A comparative phylogenetic analysis of Medicago UTNs to Arabidopsis UmamiTs, which have well characterised transport activity, combined with measurements of UTN transport activity using the *Xenopus laevis* oocyte heterologous expression system, showed that several Medicago UTNs transported amino acids. Using publicly available transcriptome resources1-3, UTN promoter:GUS fusions, qPCR, and *in situ* localisation with a UTN2-specific antibody, we confirmed that UTN genes showed specific expression patterns in nodule tissues. UTN1 (MtUmamiT144), UTN2 and UTN6 express in root hairs within hours of inoculation and expression is sustained throughout the nodule maturation process. To determine the role of UTNs in symbiosis, we generated UTN1/2/6 triple-knockout mutants using CRISPR-cas9, which show an impaired nodulation phenotype. The results indicate that amino acid transport in several nodule tissues is necessary to maintain symbiosis. UTN orthologues in other legumes also show nodule specific expression5 indicating that UTNs function in indetermintate and determinate legume species.

**References:**

[1] Schiessl, K., et al (2019) Current Biology, 29: 3657–3668

[2] Jardinaud, MF., et al (2016). Plant Physiology, 171, 2256–2276,

[3] Larrainzar, E., et al (2015). Plant Physiology, 169,.233-265.

[4] Garcia, K., et al (2023). Scientific Reports, 13, 804

[5] Frank, M., et al (2023). Nat Commun,14, 7171

**Genomic footprints and functional impact of domestication on symbiotic nitrogen fixation**

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Legume species are key components of both natural and agricultural ecosystems, owing largely to their capacity for symbiotic nitrogen fixation. Two decades of molecular and genomic studies in model systems have revealed the presence of exquisite genetic pathways that initiate symbiosis, but despite these advances we have essentially no understanding of genes that regulate symbiotic performance in the natural or agricultural environment. Here we aim to understand the evolution of symbiotic performance in the wild progenitors of chickpea and the ways in which human selection has reshaped this potential during domestication. Sequencing the genomes of >2,000 plant (*Cicer* spp) and bacterial (*Mesorhizobium*) genomes reveals the pre- and post-domestication history of plant and microbial species [1, 2], including paradoxically declining diversity in the crop and expanding diversity in the microbe. While the diversity of the plant was shaped by the wild and agricultural environments, the diversity of the microbial symbiont was shaped by bio-geographic patterns combined with widespread horizontal gene transfer. Analysis of a matrix of bacterial genotypes x host genotype combinations for symbiotic nitrogen fixation traits reveals distinct evolutionary patterns. Wild plants display patterns of local adaptation for their microbial symbionts, performing effectively in homologous but not heterologous combinations. In contrast, cultivated species are promiscuous for microbial partners, but with lower average benefit from symbiosis, consistent with a selection trade-off during domestication. Phenotyping and genetic analysis in segregating plant populations suggests genetic complexity that may involve diminishment of symbiotic capacity in the crop.

**References*:***

*[1]* von Wettberg EJB et al, Ecology and genomics of an important crop wild relative as a prelude to agricultural innovation. Nature Communications DOI 10.1038/s41467-018-02867-z (2018).

# *[2]* Greenlon A et al, Global-level population genomics reveals differential effects of geography and phylogeny on horizontal gene transfer in soil bacteria. Proc. Natl. Acad. Sci, 116:15200-15209 (2019). doi/10.1073/pnas.1900056116.

***Light sensitive short hypocotyl (LSH)* genes confer symbiotic nodule identity in *Medicago truncatula***

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Legumes grow specialized root nodules to host beneficial nitrogen-fixing bacteria that provide the plant with ammonia in exchange for carbon. These symbiotic nodules are distinct from lateral roots in morphology and function as they comprise of cells that can accommodate nitrogen-fixing rhizobial bacteria intracellularly and provide favorable conditions for the biological nitrogen fixation process. Our previous findings that the initiation of lateral roots and nodules converges at a common developmental program [1] led to the hypothesis that an additional nodule-specific program is required to determine nodule organ identity on top of the shared root-like initiation program. Recently, we have shown that two members of the *LIGHT SENSITIVE SHORT HYPOCOTYL (LSH)* transcription factor family (*MtLSH1* and *MtLSH2*), predominantly known to define organ boundaries and meristem complexity in the shoot, function as regulators of nodule organ identity. Our gain and loss of function analyses of *LSH1/2* demonstrated that these regulators are required for the development of functional nodule primordia that can support the intercellular cortical infection, the intracellular colonization, and nitrogen-fixation by the bacteria. Based on our these findings, our future work will address the question of how the coordinate recruitment of pre-existing organ identity programs, in parallel to a root initiation program, can underpin the morphological and functional divergence between lateral roots and nodules.

**References*:***

*[1] Schiessl et al., 2019, Curr Biol 29, issue 21, p. 3657-3668 .*

*[2] Lee and Orvosova et al., 2024 Curr Biol 34, issue 4  p. 825-840 .*

**Decoding CLE-SUNN-Mediated Autoregulation of Nodulation in *Medicago truncatula***

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3 Australian National University, Canberra, Australia

Autoregulation of nodulation (AON) plays a crucial role as a negative feedback mechanism in controlling the quantity of nodules formed on legume roots. Its significance lies in the fact that nodule formation and nitrogen fixation impose substantial energy costs on the host plant. In the case of the model legume *Medicago truncatula*, the AON process can be summarised as follows: (i) Rhizobial infection triggers the expression of two peptide-coding genes, MtCLE12 and MtCLE13; (ii) The mature CLE peptides migrate to the shoot and bind to their receptor SUNN; (iii) SUNN activation leads to a reduction in the expression of microRNA 2111, which acts as a negative regulator of transcript levels of TMLs (TOO MUCH LOVE); (iv) TMLs encode a Kelch repeat-containing F-box protein in the root that negatively modulates nodule numbers.

However, the downstream components of the CLE/SUNN/miR2111/TML-mediated AON mechanism remain unknown, including their role in controlling nodule formation. To address this knowledge gap, we employed RNA-Seq analysis coupled with reverse genetics to identify and characterise downstream target genes of CLE/SUNN. Here we present key target genes including novel transcription factors and genes involved in auxin homeostasis that relay AON signals and influence root nodule formation. We will propose a comprehensive model of the molecular regulation of nodulation. We aim to enhance our understanding of this intricate process, ultimately paving the way for improved sustainable crop productivity and enhanced food security.

**References*:***

[1] Imin N, et al. CLE peptide tri-arabinosylation and peptide domain sequence composition are essential for SUNN-dependent autoregulation of nodulation in Medicago truncatula. New Phytologist. 218 (1), 2023, p. 73-80.

[2] Saur I, et al. Crosstalk between the nodulation signaling pathway and the autoregulation of nodulation in Medicago truncatula. New Phytologist. 190, 2011, p. 865-874.

**The role of CLE peptides in nodulation… AND MORE!**

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The control of legume nodulation involves a complex and sophisticated molecular signalling system. CLAVATA3/EMBRYO SURROUNDING REGION RELATED (CLE) peptides are a family of small signalling molecules, including Rhizobia Induced CLE (RIC) peptides and Nitrate Induced CLE (NIC) peptides which are essential for nodulation regulation. RICs and NICs appear to act as long and short distance signals, respectively, and are triggered in response to factors that are biotic (i.e. compatible rhizobia) and abiotic (i.e. available nitrogen in the soil) [1][2]. Thus far, many CLE peptides found in legumes such as *Phaseolus vulgaris* remain uncharacterised. Here, we report on our investigations to identify additional CLE peptide family members that respond to rhizobia, available soil nitrogen, salinity stress, and plant-pathogen interactions in *Phaseolus vulgaris*. Findings provide insight into the many roles CLE peptides have in molecular signalling pathways of legumes, opening doors to understanding the evolution of this family of signalling molecules, and providing opportunities for crop improvement.

**References*:***

*[1] Ferguson BJ, Mens C, Hastwell AH, Zhang MB, Su H, Jones CH, Chu XT, Gresshoff PM (2019) Legume nodulation: The host controls the party. Plant, Cell & Environment 42: 41-51.*

*[2] Reid DE, Ferguson BJ, Gresshoff PM (2011) Inoculation- and nitrate-induced CLE peptides of soybean control NARK-dependent nodule formation. Molecular Plant-Microbe Interactions 24: 606-618.*

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| Saturday 5th October 2024Venue: Queensland Parliamentary House Annexe  |
| Time | Session | Location |
| 0730  | Registration Open | Colonnade – Level 3 - Annexe |
| Session 5 |
| Theme: **Nitrogen Fixation in Agricultural Systems**, Chairs: Dr Liz Farquarson & Georgina RobbinsLocation: Undumbi Room – Level 5 - Annexe |

**Keynote**

**Quantifying nitrogen fixation by legumes in Australia’s grain production systems at paddock-to-national scales**

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3 Brill Ag, Ganmain, NSW, Australia

Pulse and pasture legumes are key components of Australia’s grain production systems. The pulses play a critical role in breaking disease, pest and weed cycles for following cereal crops. The legumes, in a symbiotic relationship with rhizobia, also fix nitrogen (N) making them independent of fertiliser N inputs. The amount fixed can be considerable, i.e. >400 kg N/ha/year, usually obtained under ideal environmental, i.e. rainfall, conditions and with management of the legumes optimised. More commonly, annual rates of N2 fixation are 80–100 kg N/ha. The N-rich legume residues break down following harvest of the pulse grain or termination of the pasture ley to enrich the soil with mineral N and benefit subsequent crops in the rotation.

Critical in our understanding of the current and potential benefits of legumes in Australia’s farming systems is accurate accounting of rates of N2 fixation. Quantification is required at all scales for different reasons. Data at the national-to-global scales are used to identify environmental, resource efficiency and food security issues. At the other extreme, farmers are making decisions about N inputs, whether from legumes or mineral and organic fertilisers, for individual paddocks. In the space between are agronomists, microbiologists and legume breeders needing to differentiate treatment effects on legume N2 fixation across variable rainfall and/or soil environments. At all scales, the 15N natural abundance methodology for quantifying legume N2 fixation has proved to be a game breaker with Australian scientists playing a major role in optimising sampling protocols and identifying the principal sources of error of the method and how they might be managed.

**Achieving effective nodulation of legumes through HeadStart-Inoculation: inoculation of prior cereal crops to overcome constraints of dry sowing and promote commercial rhizobia establishment.**

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1 *Legume and Rhizobium Sciences, Murdoch University, WA*

2 *Department of Primary Industries and Regional Development, WA*

The agronomic benefits of successfully establishing legume crops in rotation are substantiated in southern Australian farming systems. Nonetheless, the establishment of certain legumes can be difficult, particularly with constraints to achieving a successful symbiosis with commercial inoculant rhizobia strains. It is well documented that pesticides at sowing are deleterious for rhizobia and recent constraints have emerged with the development of early season sowing into dry soils. The move towards sowing agricultural legumes to dry seedbeds has been driven by many operational factors such as increasing farm scale and the variation in the timing of the first opening rains. This, along with the continual improvements in plant production potential, machinery and management technologies, has seen the practice become increasingly viable and commonplace.

While the agronomic benefit of early sowing is accepted, it does present a legume-rhizobium symbioses establishment conundrum. A substantial proportion of the legume inoculant market is held by products that are reliant on the cool and moist conditions that typically occur following opening rainfall. The limited moisture conditions that prevail during, and following dry seeding can severely decrease the inoculant rhizobia survival and lead to reduced nodulation and poor legume yields.

This research was initiated to increase successful nodulation of legumes when establishing legumes into dry soils and remove effects of toxic contact with pesticides. Field trials are currently determining the viability of delivering inoculant products on wheat seed to achieve successful nodulation by the host legume the following year.

**Nitrogen cycling and management decision making in Central Queensland farming systems – N availability and recovery across the farming system – N impacts on productivity and implications for management in CQ**

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4 University of Queensland, Gatton, Qld Australia

Nitrogen (N) fertiliser demand for cereal cropping systems increases due to two factors:

1. Reductions in soil organic N mineralised with continued decline of natural capital; and

2. Increases in crop N demand from optimising other components of the cropping system.

The potential contribution of pulse N to the Central Queensland farming systems experiment under a contrast of cropping and fertility treatments has been modelled using the framework of Herridge (2011).

Baseline wheat, chickpea and sorghum cropping with nitrogen budgeting has a negative system N balance (-200 kg N/ha for 9 crops). Increasing legume frequency to 50% of crops has a lower negative N balance (-147 kg N/ha) but also lower dry matter inputs.

Substantial chickpea production gains have been achieved in CQ through subsurface P and K applications. These have tripled grain yields from 1.1 in untreated to 3.4 t/ha. The impact on modelled N fixed is also substantial.

**References**:

*[1] Herridge DF (2011). Managing Legume and Fertiliser N for Northern Grains Cropping. Canberra, ACT, Grains Research and Development Corporation.*

**Characterising haloalkalitolerant diazotrophs in *Technosols* eco-engineered from bauxite residue**

Song G1, You F1, Parry D2, Huang L1

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Biological nitrogen (N) fixation (BNF) driven by non-symbiotic bacteria is critical to sustain biomass productivity in the early phase of primary succession of an ecosystem, such as newly created soil-plant systems based on *in situ* soil formation for bauxite residue rehabilitation. The present study has initially characterized diazotrophic communities and key environmental factors shaping their diversity in the saline and alkaline eco-engineered bauxite-residue soil (i.e., *Technosol*). It was found that plant colonization and high organic carbon (OC) availability elevated the diversity of non-symbiotic diazotrophic communities and the BNF function in the *Technosol*. The BNF functions were closely related to the *nifH* abundance. The primary chemical factors, pH and EC, were the major drivers of the diazotrophic community structure. The total organic carbon (TOC) factor not only shaped the diazotrophic community structure, but also regulated BNF function. It is tentatively deduced that diazotrophs’ nitrogenase activity was critically up-regulated by OC supply, which may have come from decomposed organic matter (OM) and root-derived OM of colonizing plants in the early

*Technosol*. These findings provide the basis for enhancing BNF capacity in the early *Technosols* possessing moderately saline and alkaline conditions.

Has there been a rapid radiation in N2 fixing systems or is science losing its way?

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In recent years there have been an increasing reports of new N2 fixing organisms being identified, along with new plant-microbe symbioses or endophytic systems which are putatively N2 fixing, and a range of commercial inoculant products being sold as N biofertilisers and which are promoted as N2 fixing. Examples include *Gluconacetobacter diazotrophicus* with maize or wheat, *Klebsiella variicola*, *Kosakonia sacchari*, *Methylobacterium symbioticum*, and a range of other "unknown dizaotrophs” with maize, sorghum, poplar and other species. While crop yield or growth responses are often evident, do these reports herald a breakthrough in N2 fixation research and a rapid path to the holy grail, N2 fixing cereals? Unfortunately, this may not be the case with a number of simple errors leading to unsafe conclusions about the presence of N2 fixation in both laboratory and field studies. There are two principal errors made in most of these studies. The first is that N2 fixation is assumed and any evidence potentially indicative of N2 fixation is sought, ignoring many other rational explanations for the observations. The second error is that N2 fixation is not quantified, not able to be quantified, or experiments are not repeatable. Examples include the mere presence of a diazotroph as evidence for N2 fixation, or even merely a Nif gene analogue. While many studies report significant N2 fixation, they fail to actually quantity it in terms of mg/plant or kg/ha. These issues are not trivial but highlight a concerning decline in science quality which is occurring across most disciplines.

Session 6

Theme: **Nitrogen Fixation in Agricultural Systems**, Chairs: Prof David Herridge & Timothy Cameron

Location: Undumbi Room – Level 5 - Annexe

**Keynote**

**Soilborne disease of pulse crops: constraints to nodulation and N2 fixation**

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Historically viewed as a break crop to reduce weed and disease issues in cereal crops, pulses are now often highly profitable in their own right and grown in frequent rotation. As cropping frequency increases, soilborne disease is emerging as an important constraint to pulse crops[1].

In 2022, we conducted a survey of 76 paddocks on Kangaroo Island of either lupin or bean to determine the distribution of key pulse root pathogens and nitrogen-fixing rhizobia in soil (pre-sowing) using PREDICTA® Research qPCR assays. Crop root health, including disease and nodulation, was assessed for 50 paddocks in spring. Soil analysis showed common were widespread and less common, but highly pathogenic species, *Aphanomyces euteiches* and *Phytophthora megasperma,* were detected in 5-13% of paddocks. Rhizobia numbers were low or below detection in more than half of paddocks.

Average visual root health score (0-5 scale) ranged from 1.7-4.9 indicating wide variation in root health; however overall root disease was common and significant. There was a significant negative correlation between root disease and nodulation (P<0.001). This survey is complimented by results from six replicated field trials conducted in southern Australia on lentil and chickpea between 2019-2021. These trials evaluated a range of seed chemical treatments to control soilborne pathogens on root health, nodulation and N fixation. Nodulation was increased by nearly 50 % at two sites and N fixation and one site. This work provides promising evidence that fungicides with low to no toxicity to rhizobia may provide improvements in nodulation.

**References*:***

*[1] Gontar et al., The health report – emerging pulse root diseases, GRDC Adelaide Update, 11 Feb 2020.*

**Developing new harvestable Aerial Seeded Pasture Legumes (ASPL’s) to reduce synthetic nitrogen reliance in cropping systems.**

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Biological nitrogen (N) fixation from legumes can reduce the over-dependence on synthetic N fertiliser in crop production systems of Western Australia (WA). Over the last 30 years the adoption of novel aerial-seeded pasture legumes (ASPLs) into WA farming systems have demonstrated sustainable and profitable benefits. Many ASPL’s can be harvested easily with conventional harvesters on farm, then cheaply and reliably multiplied by seeding dry in summer or autumn to exploit favourable conditions early in the season. These legumes enhance N fixation (free N farming), increase the yield and protein of following cereals and oilseed crops, therefore ultimately reducing fertiliser N reliance.

The recently formed Harvestable Annual Legume Options (HALO) project is expanding research into all areas of WA agro-ecological zones to evaluate a range of potential ASPL’s. It is selecting and evaluating those which possess diverse maturities, contain either hard and soft seeded characteristics and be adapted to different soil types. Three major aims of the project are to provide growers in the target environments with greater harvestable ASPL’s cultivar choices to increase adoption. Secondly to optimise the symbioses through effective inoculation of commercial rhizobia strains which will run parallel to the cultivar development, such as HeadStart-Inoculation®. Thirdly, before cultivar release, evaluate the viability of the introduced legume in crop rotation scenarios through field measurements to capture N balance, soil water usage, and adverse weed implications. Importantly, project outcomes will assist growers and advisers in assessing which ASPL’s should be implemented in specific agro-ecological zones to best manage risk whilst increasing profit.

**The role of mineral nitrogen on mungbean nodulation**

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Previous research has described poor nodulation in Australian mungbean crops and called for work on varietal differences in nodulation in response to soil nitrate levels. A growth pouch-based study was undertaken using two mungbean genotypes (AVTMB#4 and Jade-AU) with and without a starter nitrogen (N) dose 0, 1, 2.5, 5, and 10 mM. Nodule score, mass and %Ndfa (plant N derived from atmospheric N) were inversely linearly correlated to added nitrate levels. The 10 mM nitrate treatment was associated with a reduction of 52%, 45% and 42% in nodule score, nodule mass and %Ndfa, respectively, after 25 days of growth, relative to N-free control treatment (0N) (averaged across both genotypes). Jade-AU was more sensitive to nitrate than AVTMB#4, with nodule score, nodule mass and %Ndfa decreased by 28, 16 and 41%, respectively, relative to 0N following 25 d of growth on 10 mM applied nitrate. No influence of starting nitrogen (1 mM nitrate) on nodulation or plant development was detected. A method for rapid screening for nitrate sensitivity for biological N fixation for Australian mungbean varieties is suggested, using growth pouches and a qualitative nodule scoring method.

**Understanding the nitrogen contribution of chickpea in Queensland using the natural abundance technique**

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Chickpeas are an important rotation crop for both winter and summer grain production across Queensland. They can contribute to nitrogen through atmospheric fixation, but the magnitude is dependent on cropping intensity and fallow management. Agronomic practices related to chickpea production have increased grain harvest index, making the amount of nitrogen exported in grain relative to total crop uptake, shift also.

In 2023, twenty-five chickpea crops across five subregions of Queensland were monitored for biomass and grain yield. Sites had soil samples taken at sowing, harvest and at the sowing of the subsequent crop to characterise soil fertility and water.

Above ground dry matter at maturity ranged from 850 to 7500 kg/ha, containing between 15 and 137 kg N/ha. Nitrogen derived from atmosphere (Ndfa) values ranged from 11 to 69%, contributing between 8 and 59 kg N/ha.

Group 2 herbicides severely impact the biological nitrogen fixation of French serradella

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Pasture legumes are crucial to the sustainability of ley farming systems. They provide a disease break for cereals, nutritious feed for livestock and supply biologically fixed nitrogen (N) for subsequent non-leguminous crops. However, there are frequent observations where biological N fixation from the pasture phase is significantly less than expected. We term this an N fixation deficit. Previous field experiments have indicated that the application of group 2 herbicides contributes to this N deficit. Pre-emergent and early-season post-emergent herbicides are applied to avoid early-season weed competition. Group 2 herbicides, inhibitors of the aceto-lactate synthase (ALS) system, are widely used in the farming systems of the Western Australia wheatbelt to control weeds.

Screen-house experiments were conducted to quantify the damage of group 2 herbicides to French serradella (*Ornithopus sativus*)cvs. Frano and Margurita inoculated with commercial strain WU425. Imazethapyr, imazamox and flumetsalem were applied at logarithmic rates to ascertain a dose-response curve and elucidate the tolerance of these cultivars at three different crop stages.

Root nodulation of both French serradella cultivars was more sensitive to herbicide damage than above-ground biomass. An example is imazethapyr applied post-emergent, which reduced nodulation by 50% when applied at 40% of the recommended label rate (140g/ha). To reduce the top dry weight by 50% required applying imazethapyr at 170% of the recommended label rate. The implication of this is that although the legume may be observed as unaffected by these herbicides, N fixation is being severely affected.

**Poster Presentation Abstracts**

**A novel nucleotide-binding domain leucine-rich repeat receptor (NLR) involved in soybean nodulation**

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Legumes can exploit their relationship with nitrogen-fixing rhizobia to thrive in low nitrogen environments via the formation of symbiotic organs on their roots, termed nodules. The plant innate immune system has been shown to be a major determinant of nodule formation. For example, soybean nucleotide-binding domain leucine-rich repeat receptors (NLRs) have been shown to restrict symbiosis with certain strains of rhizobia upon recognition of specific rhizobia proteins. Here, we report the characterisation of a novel NLR of soybean which responds to Nod factors unlike previously reported NLRs. Interestingly, promoter::*GUS* fusions revealed this gene is induced from early infection through to mature nodules. When overexpressed, the *NLR* reduces nodule number, yet when knocked-out via CRISPR genome editing, there is no change in nodule number. mRNA of the *NLR* undergoes alternative splicing to produce at least four different protein isoforms. NLRs in plant-pathogen interactions, often called *R*-genes, have recently been shown to form tetrasomes capable of degrading nucleotides and facilitating cell death. Sequence analysis suggests the soybean NLR involved in nodulation has the necessary catalytic sites for this enzyme activity. New insight into this gene and our current understanding of the interplay of plant immunity in legume nodulation will be presented.

**Pan-genomics and -transcriptomics of symbiotic nitrogen fixation in mungbean**

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Legumes can thrive in low nitrogen environments due to their ability to utilize atmospheric di-nitrogen via symbiotic nitrogen fixation (SNF) by rhizobia housed in legume root nodules. SNF is a complex plant trait with over 200 genes found to contribute substantially in a few model species. However, SNF varies between and within a species meaning the goal of improving SNF in legumes will require work on collections of diverse genotypes, to understand the genetic potential for improvement within each target species. To facilitate this for the tropical legume, mungbean, we have begun to characterize the pan-genome and transcriptome from the point of view of nodulation and SNF. Mungbean’s short life cycle makes it an ideal, high value component of existing cropping systems that provides soil N enrichment amongst other benefits. To begin to explore the natural variation of SNF in mungbean, eight diverse genotypes were selected to compare SNF-related traits, including biomass, nodulation phenotypes, N derived from the atmosphere (Ndfa) and gene expression. 15N isotope analysis allows us to determine %Ndfa and total Ndfa providing an estimate for SNF effectiveness. Comparative transcriptome analysis is being undertaken to investigate SNF gene expression in these different genotypes. Together, these results will provide a foundation to understand the SNF capabilities of diverse mungbean accessions. This, coupled with Genome Wide Association Studies in hundreds of diverse mungbean genotypes, will advance our understanding of the genetic basis of SNF and its improvement via predictive plant breeding and genome editing in mungbean.

**Exploring the Effects of Soil Acidity on Root Nodulation and Nitrogen Fixation in Chickpeas**

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Chickpea (*Cicer arietinum*) is a major legume crop. The optimum soil pH range for chickpea growth is between 6.0 – 8.0. Soil acidity (pH<5.5) negatively impacts plant growth and causes nutrient deficiencies and toxicities. Previous studies hypothesize that, in legumes, soil acidity may disrupt the chemical signaling between host plants and rhizobia and reduce rhizobial colonisation, leading to reduced nodulation[1]. However, such studies are lacking in chickpeas. This project aims to evaluate the nodulation and nitrogen fixation of various cultivated chickpea varieties under acid soil conditions. Furthermore, it will identify the nodulation stages that are affected by soil acidity and the factors contributing to reduced nodulation and nitrogen fixation. Around 75 cultivated chickpea varieties will be grown in river sand in glasshouses under two different pH ranges (4.5 - 5.0 and 6.0 - 6.5). Initially, a general screening will be performed in this setup to identify varieties that are nodulating better under acid soil. Two varieties with contrasting nodulation ability under low pH conditions would be used to perform detailed nodulation time-course experiments to identify the nodulation stages that are affected by soil acidity and to observe the changes in nodule anatomy, flavonoid production, and *nod* gene induction in rhizobial symbiont, under acid soil conditions. Overall, this study will identify the chickpea cultivars best suited to low pH conditions and provide breeders with options for improving chickpea nodulation and yields on acid soils.

**References*:***

*[1] Ferguson, B. J. et al., 2013, Plant signaling & Behavior, 8(3), e23426.*

**Tyrosine Sulphated Root Meristem Growth Factor Peptides Regulate Root and Nodule Development in Soybean**

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Legumes form a beneficial symbiotic relationship with soil bacteria that can fix atmospheric nitrogen into a plant usable form, resulting in a reduced reliance on synthetic nitrogen fertilizer and the associated negative environmental issues. This process is known as nodulation and involves complex molecular signaling pathways to control the physiological changes that are required to initiate and maintain nodule organogenesis. We have identified novel tyrosine sulfated peptides, called Root Meristem Growth Factor (RGF) peptides, in one of the most widely produced legumes, soybean (*Glycine max*)1. They are homologous to

Arabidopsis RGF/GLV/CLEL peptides that play essential roles in regulating meristematic activity and immune responses2. We have identified conserved orthologs in other agriculturally important legumes, including *Phaseolus vulgaris*, *Pisum sativum* and *Lotus japonicus.* Within the soybean gene family, we have functionally characterized five soybean RGF-peptide encoding genes using multidisciplinary molecular biology techniques. Our results identified that theyareexpressed in the root tip region where meristem is located and during different growing stages of lateral root growth and nodulation. They also demonstrate a systemic signal transduction pathway, which is not seen in Arabidopsis and necessary for proper root growth and nodule development. These findings enhance our understanding of legume signaling and symbiotic nitrogen fixation and benefit future legume crop development programs which will improve the impact agriculture has on the environment, human health, economy, and biodiversity.

**References:**

[1] Hastwell et al. 2024, TIPS accepted, PLANTS-D-24-00070

[2] Matsuzaki et al., 2010, Science, 329, 1065-1067

**Correlating Flavonoid Exudation and Microbiome of *Medicago truncatula***

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Symbiotic nitrogen fixation in legumes provides a sustainable nitrogen source for agriculture. However, the symbiosis is often not optimal. A potential method to improve the symbiosis is by modifying the exudation of flavonoids, a family of metabolites that signal the symbiotic infection of rhizobia. This poster reports on an experiment that utilised metabolomic and metagenomic methods to test for a correlation between the microbiome in three spatial compartments at different distances from the root of a null segregate *Medicago truncatula* (2HA) and an isoflavone synthase overexpression transgenic line (IFS:OE), which showed different flavonoid exudation when grown on media (Liu *et al.* 2017). Plants were grown in a mixture of sand and field soil, and nodulated with native soil rhizobia, with significant increases in total shoot N in the IFS:OE line compared to the control. The compartments harvested were bulk soil, rhizosphere soil and root tissue. The experiment successfully identified differences in the microbiome of the compartments, with the diversity of microbes decreasing with proximity to the root. However, the differences in flavonoid exudation observed when 2HA and IFS:OE were grown in media were not observed when grown in soil, with a difference in flavonoid accumulation only observed within the root. This result meant a correlation between flavonoid exudation and microbiome could not be made, as there was no difference in either flavonoid profile or microbiome outside of the root when comparing plant lines. These results suggest a greater understanding of the root exudation mechanism is necessary to manipulate nodulation through flavonoid exudation.

**References*:***

*[1] Liu Y.* *et al, Ethylene signaling is important for isoflavonoid mediated resistance to Rhizoctonia solani in Medicago truncatula. Molecular Plant-Microbe Interactions vol. 30: 2017, p. 691-700*

**Exploring the mungbean pan-genome**

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Mungbean (*Vigna radiata*) is a short-season tropical pulse grown on over six million hectares worldwide, mostly in Southern Asia with increasing importance in Australia and East Africa. Current commercial varieties show little genetic diversity with breeding focussed on a limited number of agronomic traits like disease resistance and flowering time. Luckily, we have access to diverse core collections and an Australian NAM (Nested Association Mapping) population.

Eight genetically diverse NAM parent accessions were selected based on available DArTseq SNP data including commercial varieties and an Australian wild accession (*V. radiata* var. *sublobata*). High molecular weight DNA was extracted for long-read HiFi PacBio sequencing (71-136x coverage). These reads were assembled into high quality genomes (contig N50 17.632.8 Mb) with a total assembly length varying from 512.8 to 577.2 Mb and a BUSCO completeness score of over 99% for each of the eight accessions. RNAseq data was generated using Illumina paired-end sequencing for shoot, root, and nodule tissues to allow for evidencebased gene annotation and differential gene expression analysis. Overall, this approach allowed for improved assembly statistics relative to the publicly available genomes VC1937A and Vrad\_JL7.

This high-quality pan-genome makes it possible to investigate diverse topics from crop improvement to evolutionary biology. It can be utilised by researchers and breeders to explore and improve complex traits like nitrogen fixation, yield stability, seed quality and adaptability to climate stresses. Integration of this sequence data with other data types like diverse phenotypes will help us move towards genome-based predictive breeding and crop improvement.

**Identifying Drought-Tolerant QTLs for Nitrogen Fixation in Cowpea and *Lotus japonicus* Using GWAS**

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Legumes fix atmospheric nitrogen through a symbiotic relationship with rhizobium, enhancing soil nitrogen levels and reducing the reliance on synthetic fertilizers. However, abiotic stresses such as drought disrupt this process, negatively impacting nitrogen fixation and overall yield [1]. Increased frequency of droughts due to climate change has further diminished nitrogen fixation in legumes, forcing farmers to rely more on synthetic fertilizers to maintain crop yields. This dependence contributes to carbon emissions and poses sustainability challenges. The presence of genetic variation in nitrogen fixation response to water deficits among legume cultivars presents an opportunity to enhance drought tolerance in nitrogen fixation. Integrating these variations into modern crop breeding programs could provide a sustainable approach in addressing this challenge.

Phenotyping for nitrogen fixation under drought conditions of a cowpea MAGIC population (300 lines) [2] and a wild *Lotus japonicus* germplasm (140 lines) [3] will facilitate the identification of drought-tolerant lines. Drought stress will be induced using the gravimetric method, and key traits such as nitrogen fixation by measuring 15N incorporation into pheophytin [4], leghemoglobin concentration, nodule number, biomass, chlorophyll concentration, and plant height will be assessed at harvest after drought treatment. The collected data will be used in Genome-Wide Association Studies (GWAS) to identify Quantitative Trait Loci (QTLs) associated with drought tolerance. These QTLs will subsequently be validated by gene editing using CRISPR and other detailed molecular studies. QTLs which are found to be imparting tolerance can be incorporated into future legume breeding programs.

**References*:***

*[1] Serraj et al. (1999*). J. Exp. Bot*. 50: 143-155. doi: 10.1093/jxb/50.331.143*

*[2] Huynh et al. (2018).* TPJ. *93: 1129-1142. doi: 10.1111/tpj.13827*

*[3] Shah et al. (2020).* Nat Commun*11, 253. doi: 10.1038/s41467-019-14213-y*

*[4] Kahn et al. (2002).* Anal. Biochem. *307, 219-225 doi: 10.1016/S0003-2697(02)00046-5*

Assessing the interaction between an acid-tolerant strain of rhizobia and faba bean.

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The process of nitrogen fixation requires an adequate number of effective rhizobia be present in the soil surrounding the legume. This enables nodulation of the plant’s root system. In acid soils, adequate numbers of rhizobia are unlikely to be present. The rhizobia that nodulate faba and broad bean are especially sensitive to soil acidity below pHCa 5.5. As such, the expansion of faba and broad bean sowing into areas with acid soils is currently restricted. Trials have been conducted in recent years Australia-wide to evaluate the performance of several high-performing acid-tolerant rhizobia strains. This included strain SRDI-969 short-listed by the South Australian Research and Development Institute (SARDI). The aim of these trials was to deliver strains to industry that increase grain legume adaptation and production, especially in acidic soils. High-performing strains were assessed in both acidic and non-acidic soils. This included comparing the performance of SRDI-969 to the Australian commercial inoculant group E/*F* strain, WSM1455, in an alkaline soil at Tamworth (NSW 2340) in 2022. Where seed was directly inoculated with either strain of rhizobia, roots of plants inoculated with SRDI969 had a significantly higher number of nodules than the roots of plants inoculated with the commercial strain WSM-1455. Yields of crops inoculated with either strain were not significantly different. Results from Tamworth in 2022 contributed towards the commercial release of the acid-tolerant strain SRDI-969 to growers for the 2024 season. Studies to improve our understanding of nitrogen fixation in pulses in Australia continue.

**References*:***

*[1] Acid tolerant rhizobia improve nodulation of faba and broad bean. New Group F Rhizobia Inoculant for Faba and Broad Bean Fact Sheet. Grains Research & Development Corporation.*

**Impact of inoculation technology on nodulation and nitrogen fixation of chickpea and mungbean under different nitrogen management options**

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In Australia’s northern grains region, chickpeas and mungbeans are critical legume crops because of their significant economic and biological contributions. Growers utilise commercial rhizobia inoculants CC1192 for chickpea and CB1015 for mungbean aiming to enhance nodulation and nitrogen (N) fixation under variable soil conditions and agronomic management practices.

Increasing soil mineral N reduces the N fixation of chickpea and mungbean. Different fallow lengths based on cropping intensity across the northern region also change the soil mineral N levels at sowing of the pulse crops. An experiment at Wellcamp (near Toowoomba) has been established to explore the interaction between cropping intensity/fallow length and soil mineral N status on chickpea and mungbean biomass, grain yield and N fixation. Screening of new rhizobia strains for emerging chickpea regions in southern and western Australian is embedded within the design. For mungbeans, the efficacy of peat inoculation vs water injection inoculation practices is explored.

This research will enhance the growers understanding of the role of nitrogen fixation by chickpeas and mungbeans as part of optimising their system nitrogen management.

**Identifying transport proteins important for symbiosis**

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Efficient nitrogen fixation in the legume: rhizobia symbiosis relies on nutrient exchange between the two partners. The plant provides mineral nutrients and photosynthates as an energy source in exchange for fixed nitrogen. Transport of sugars, metals, sulphate and a range of other mineral nutrients into the nodule is required to support rhizobia which are enclosed within the symbiosome membrane in infected cells, while the products of nitrogen fixation must be transported out of the nodule to the rest of the plant. The architecture of the nodule means that a number of cell layers including vasculature, cortex, endodermis and infected or uninfected cells within the infected region must be traversed. While this movement of compounds may include symplastic or apoplastic pathways, facilitated transport across cell membranes and particularly into and out of the symbiosome is also required. To identify novel transport proteins potentially important for symbiosis we have mined publicly available nodule RNAseq [1] and single cell transcriptome datasets [2], as well as our symbiosome membrane proteome. Potential dicarboxylate, metal, amino acid and peptide transporters have been identified. *Lotus japonicus* LORE lines for a number of these candidates have been screened to identify those where nitrogen fixation is compromised and the candidates will be further studied to determine their role in nodule function and nitrogen fixation including cellular and intercellular location, and their substrates.

**References*:***

*[1] https://lotus.au.dk/expat/*

*[2] Frank M. et al, Single-cell analysis identifies genes facilitating rhizobium infection in* Lotus japonicus*, Nature Communications, vol. 14, 2023, p. 7171*

**Effect of different carbon substrates in growth media on desiccation tolerance of rhizobia**

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Peat extract preconditions rhizobia, improving their desiccation tolerance. However, the properties of peat that affect desiccation tolerance are not well understood due to its complexity. To better understand how growth media influence the growth, survival, and metabolome of rhizobia, we modified minimal defined media with different C sources and C:N ratios. GC-MS analysis followed by untargeted metabolomic analysis identified 145 metabolites in the rhizobia after growth in different C sources. Multivariate analysis revealed that C source in growth media had a significant effect on rhizobial metabolome. Trehalose, 3-hydroxybutyric acid and 5,6-dihydrouracil contributed to the highest variation in rhizobial metabolome. Media containing L-arabinose significantly improved both rhizobial numbers and trehalose accumulation at early stationary phase as well as enhanced survival after airdrying on polyethylene beads. In contrast, mannitol and succinate had a more variable effect on survival depending on rhizobial strain. Lower C:N ratios improved the survival of rhizobia on beads during storage, but the effect was not as great as modifying C substrate. Pearson correlation analysis revealed that survival immediately after drying is positively linked with intracellular trehalose accumulation. Also, survival after one week of storage positively correlated with the metabolites 3-hydroxybutyric acid, glycolic acid, and methylmalonic acid. The results highlight the significance of C substrate quality on rhizobial metabolism and subsequent survival. We hypothesise that improved survival of rhizobia in peat extract may be a result of the type of C compounds present which will inform further research.

**References*:***

*[1] Atieno M. et al, 2018, Aqueous peat extract exposes rhizobia to sub-lethal stress which may prime cells for improved desiccation tolerance,* *Appl. Environ. Microbiol., vol. 102, no. 17, p 7521-7539.*

*[2] Casteriano A. et al, 2013 Physiological changes in rhizobia after growth in peat extract may be related to improved desiccation tolerance, Appl. Environ. Microbiol., vol. 79, no. 13, 2, p 3998-4007.*

*[3] Feng L. et al, 2002, Morphological changes of rhizobia in peat cultures, Appl. Environ. Microbiol., vol. 63, no. 3, p 1064-1070.*

**The effects of rhizobial nodulation and AM fungal colonisation on the nutritional quality of grain legume crops**

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Deficiencies in nitrogen (N), phosphorus (P), zinc (Zn) and iron (Fe) in agricultural soils can greatly harm crop yield and quality, while reducing nutrition for human health [1]. Pulse crops are vital in sustainable agriculture due to their ability to fix atmospheric N through symbiosis with rhizobial bacteria [2]. Arbuscular mycorrhizal (AM) fungi also benefit legumes by acquiring essential nutrients like P, Zn and Fe for the host plant [3]. However, the presence of phytic acid (PA), the storage form of P in seeds, chelates with Zn and Fe, reducing their bioavailability for humans [4]. There is limited research on their impact of rhizobia and AM fungi on PA accumulation, micronutrient bioavailability, and protein content in legume crops.

Our study investigated the interaction between rhizobia and AM fungal inoculation (with *Rhizophagus irregularis*) in four legume species using three growth substrates. We found that AM colonisation reached up to 37.5% in the roots of field pea and lentil, correlating with increased nodulation. A subsequent experiment assessed nutrient accumulation and bioavailability in the seeds of five field pea cultivars, comparing plants with and without *R. irregularis* inoculation. The results showed that AM fungi enhanced belowground biomass but slightly reduced aboveground biomass in all five varieties at maturity, with no effect on the pea dry weight. Seed phytic acid and protein content, and micronutrient bioavailability are yet to be analysed.

This research underscores the potential for synergistic interactions between rhizobia and AM fungi to enhance the nutritional quality of legume crops, promoting more sustainable and efficient agricultural practices.

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