# final report

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# Rhizobia survival and new methods to improve nodulation in tropical legumes

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# Abstract

This project aimed to test the effectiveness of introduced and native rhizobia in two tropical pasture legumes (Stylosanthes seabrana or Caatinga stylo and Desmanthus virgatus) and evaluate new approaches with the potential to improve rhizobia establishment and legume nodulation for improved nitrogen fixation and greater legume growth. Glasshouse trials were conducted on a range of collected pasture soils to assess the legume species compatibility and nodulation potential of native rhizobia. Field trials to measure the impact of introduced rhizobia using new inoculation approaches in the hot, dry environments in which perennial tropical legumes are typically sown were also conducted. The results of this project demonstrated that for both legume species, inoculation using commercial rhizobia strains is a well-founded recommended practice and that methods that deliver inoculum deeper into the soil such as water injection or granules are more likely to be effective than inoculum on seed that is sown shallow. Of the 14 soils assessed in the glasshouse, five soils showed significant increases in nodulation due to the addition of the commercial inoculum strain (CB3481) for Caatinga stylo with five others showing increases. Four of those soils also yielded an increase in plant growth in Caatinga stylo where inoculum was added (an important result given it is not always easy to show responses due to the inherent soil fertility masking or reducing nitrogen fixation in the initial stages of pasture establishment and growth). Three soils had higher nodulation with the addition of the commercial inoculum strain (CB3126) for desmanthus but this did not translate into an increase in plant growth and may indicate that more research is required to identify a more effective rhizobium strain for this species. This study shows that for many of soils of the brigalow bioregion, inoculation with an effective rhizobia strain that is specific to the legume species is critical. Even if native strains do form nodules with either species, there is no commercially available method to test a soil for the presence of compatable and effective rhizobia strains. It is also therefore very important that introduced commercial strains survive the inoculation and planting processes so that enough rhizobia are present when the seed germinates.

# Executive Summary

Declining sown pasture productivity as a result of reductions in plant available soil nitrogen is an ongoing constraint to grazing production across the brigalow bioregion of central and southern Queensland and northern New South Wales (Peck et al. 2011). Research and industry consultation indicate a 50% decline in pasture productivity across northern Australia since initial development, with an estimated economic impact of over $17B over the next 30 years. Research suggests that legume establishment offers the most cost effective long-term remediation strategy for improving pasture quality and yield (Peck et al. 2011). Legume survival, growth and contribution to soil nitrogen are however often reduced by the effectiveness of currently applied methods of rhizobia inoculation that limit effective nodulation of legumes. Improvement of inoculation practices could significantly lift productivity across the northern beef industry.

Two adapted tropical legumes (Stylosanthes seabrana or Caatinga stylo and Desmanthus virgatus) have been found to persist in sown grass pastures within central and southern Queensland. Methods to successfully establish both species into existing pastures are being investigated in the MLA project “Improving productivity of rundown sown grass pastures” (B.NBP.0639). Extension and on-farm research activities within the project have captured grazier interest and supported many producers to re-invest in pasture legumes. Through this work, an opportunity to assess new approaches to establishing rhizobia to ensure effective nodulation was recognised with the intent of developing recommendations to ensure maximum impact for investment in legumes over the coming years.

This project aimed to test the effectiveness of introduced and native rhizobia in tropical pasture legumes and evaluate new inoculation approaches with the potential to improve rhizobia establishment and legume nodulation for improved nitrogen fixation and greater legume growth. Glasshouse trials were conducted on a range of collected pasture soils to assess the legume species compatibility and nodulation potential of native rhizobia. Field trials were also conducted to measure the impact of introduced rhizobia using new inoculation approaches in the hot, dry environments in which perennial tropical legumes are typically grown.

Soils from 15 grass-only pastures in central Queensland, southern Queensland and northern New South Wales were collected and used in glasshouse trials to evaluate the capacity of native rhizobia to form effective nodules on Caatinga stylo and desmanthus. Caatinga stylo in particular was considered to have specific rhizobia requirements. Comparisons with both an introduced inoculum strain and the addition of nitrogen fertiliser were carried out. Two trials were conducted because while the first provided some useful insights, suspected contamination of the uninoculated control pots necessitated a second trial to permit conclusions to be drawn with confidence.

Of the 14 soils assessed in the glasshouse, five soils showed significant increases in nodulation due to the addition of the commercial inoculum strain (CB3481) for Caatinga stylo with five others showing non-significant increases. Four of those soils also yielded an increase in plant growth in Caatinga stylo where inoculum was added (an important result given it is not always easy to show responses due to the inherent soil fertility masking or reducing nitrogen fixation in the initial stages of pasture establishment and growth). Three

soils had significantly higher nodulation with the addition of the commercial inoculum strain (CB3126) for desmanthus but this did not translate into an increase in plant growth and may indicate that more research is required to identify a more effective rhizobium strain for this species. This study shows that for many soils of the brigalow bioregion, inoculation with an effective rhizobia strain that is specific to the legume species is critical. Even if native strains do form nodules with either species, there is no commercially available method to test a soil for the presence of compatable and effective rhizobia strains. It is also therefore very important that introduced commercial strains survive the inoculation and planting processes so that enough rhizobia are present when the seed germinates.

Field trials were conducted in two different environments and soil types in southern Queensland to compare the relative effectiveness of different rhizobia establishment strategies in difficult conditions (i.e. small seeds, hot and dry summer) that are common when establishing sub-tropical pastures. While weather played a significant role by making establishment difficult with these trials, some important results have been obtained.

The first of these trials was on a heavy clay soil at Warra, on the Darling Downs. It was established with identical treatments in both rainfed and irrigated sections. The second was a rainfed trial planted at Kindon, on the southern Downs, on a loam soil. Unfortunately, due to circumstances beyond the control of the project team, only one sampling has been carried out at this site with no nodulation being detected on any plants for any treatments. The project team is committed to continuing maintenance of the trial after the project conclusion so that follow-up sampling and results can be gained from the hard work already invested during the project term.

The results of this project demonstrated that for both legume species, inoculation using commercial rhizobia strains is a well-founded recommended practice and that methods that deliver inoculum deeper into the soil such as water injection or granules are more likely to be effective than inoculum on seed that is sown shallow.

# Table of Contents

# 1 Background

Declining sown pasture productivity as a result of reductions in plant available soil nitrogen is an ongoing constraint to grazing production across the brigalow bioregion of central and southern Queensland and northern New South Wales (Peck et al. 2011). Research and industry consultation indicate a 50% decline in pasture productivity across northern Australia since initial development, with an estimated economic impact of over $17B over the next 30 years. Research suggests that legume establishment offers the most cost effective long-term remediation strategy for improving pasture quality and yield (Peck et al. 2011). Legume survival, growth and contribution to soil nitrogen are often reduced by the effectiveness of currently applied methods of rhizobia inoculation that limit effective nodulation of legumes (O'Hara et al. 2014).It is therefore likely to be the case for Caatinga stylo and desmanthus. Therefore, a great opportunity exists in which legume inoculation practices can be improved and in doing so, lift productivity across the northern beef industry.

Consultations with members of the pasture seed industry have revealed that many believe that inoculation of tropical legumes is ineffective and unnecessary, especially as legumes with small seeds are left on the surface of very hot soils for days or weeks before germinating rains arrive. These are difficult conditions for rhizobia to survive. However, research indicates that effective rhizobia establishment and inoculation has the potential to triple legume productivity (Clem and Jones 1996). Any new approaches that improve inoculation in these difficult conditions will dramatically improve subsequent pasture quality and quantity, red meat production and profitability across the vast areas of grass only pastures in southern and central Queensland, and into northern New South Wales. There are approximately 30 million hectares of sown grass pastures in Queensland alone (Peck et al. 2011). These vast swathes of land hold great potential for increased production through legume augmentation however, such potential will never be realised if inoculation practices are not successful.

The MLA project "Improving productivity of rundown sown grass pastures" ((B.NBP.0639) has confirmed that adapted tropical legumes (e.g. Caatinga stylo, desmanthus) will persist when sown on appropriate soils in the brigalow bioregion and is evaluating methods to establish them into existing pastures. Extension and on-farm research activities in the project are capturing graziers' interest and supporting many to re-invest in pasture legumes. Consequently, there is a great opportunity to assess new approaches to establishing rhizobia to ensure effective nodulation and develop recommendations that will ensure maximum impact for this investment in the coming years.

This project aimed to test the effectiveness of rhizobia in sub-tropical pastures and evaluate new approaches with the potential to improve rhizobia establishment and legume nodulation for improved nitrogen fixation and greater legume growth. Glasshouse trials assessed the effectiveness of native rhizobia in soils collected from across the region. Field trials assessed new inoculation approaches in the hot, dry environments in which perennial tropical legumes are typically sown. These trials and their results will be discussed with graziers participating in the afforementioned "Improving productivity of rundown sown grass pastures" project (B.NBP.0639) to determine the most practical methods for commercial properties and develop recommendations for their use across the region. The results and outputs of this project will also be shared with participants of the sub-tropical pastures project within the new MLA Southern Feedbase initiative, which identified rhizobia and inoculation as critical areas for future RD&E investment.

Previous findings by research scientist Richard Date (manuscript in preparation for publication) showed that deep placement of inoculum either on a preceding cereal crop or on inert plastic beads at the time of sowing Caatinga stylo provided more nodules than when inoculum was introduced on the seed of the surface sown legume. Date concludes that inoculation of surface sown seed for Caatinga stylo is a risky practice where surface soil temperatures are high (up to 50 $^{o}C$) and dry at the time of sowing. This project aimed to test some new rhizobia establishment techniques that have been developed for grain crops with tropical legumes.

# 2 Projective objectives

## 2.1 By the 31st July 2015

1. Evaluate the potential of native rhizobia from grass-only pastures/soils in central Queensland, southern Queensland and northern New South Wales to form effective nodules on key legume species (including Caatinga stylo and desmanthus) that are currently considered to have specific rhizobia requirements.
2. Compare the relative effectiveness of different rhizobia establishment strategies in difficult conditions (i.e. small seeds, hot and dry summer) that are common when establishing sub-tropical pastures.
3. Produce guidelines and specific recommendations to establish rhizobia with legumes in tropical and sub-tropical pastures.

### 2.1.1 Additional detail

Potential of native rhizobia to form effective nodules on key legumes (2012/2013)

1. Topsoil was collected from 14 representative sub-tropical pastures in southern Queensland, central Queensland and northern New South Wales (with support from the southern Feedbase Initiative).
2. A replicated pot trial was used to assess the growth, nitrogen content, nodulation and nodule effectiveness of legumes grown in these soils.
3. Each soil included an ‘inoculated’ and ‘uninoculated’ treatment.
4. Key legumes included commercially available desmanthus and Caatinga stylo.

Rhizobia establishment methods (2013/2014/2015)

1. Replicated field trials were conducted and managed by DAF to test and compare potential new methods to establish rhizobia in sub-tropical pastures. Two legume species were used, Caatinga stylo and desmanthus.
2. Two trial sites in south Queensland were used. One on a heavier clay soil (ideally with irrigation potential), while the second site was on a lighter clay loam.
3. Five nominal treatments were tested and compared: nil, traditional peat coating, commercially inoculated and coated seed, water injection and clay granules.
4. Key measures included: soil fertility, legume growth and nitrogen content.

Rhizobia establishment guidelines and recommendations (2014/2015)

1. Trial treatments, emerging results and implementations will be discussed, developed and reviewed with graziers groups within the “Improving productivity of rundown sown pastures” project (B.NBP.0624). This will provide engagement with grazier groups and provide momentum for extension and on-farm testing of promising options.
2. Recommendations to establish rhizobia in tropical and sub-tropical pastures will be developed and collated into a short technical leaflet for use by graziers and their advisors (including seed company representatives).
3. This technical leaflet will be distributed to grazier groups participating in the concurrent “Pasture rundown project” (B.NBP.0639), the sub-tropical pastures project within the Southern Feedbase Programme and other interested graziers.

# 3 Methodology

## 3.1 Glasshouse trials to evaluate the effectiveness of native rhizobia on Caatinga stylo and desmanthus

Two glasshouse pot experiments involving 15 different soils from Queensland and northern New South Wales were conducted. Soil collection sites were selected to cover the major soil types suitable for pasture improvement across southern and central Queensland. Two sites were also sampled from northern New South Wales to link with research being conducted on sub-tropical legumes in that region. A northern site located at Hughenden was selected due to the high incidence of the leguminous native sensitive plant (*Neptunia gracilis*). This species is thought to nodulate with a similar rhizobia strain to that produced commercially for desmanthus, hence the importance of including soil from this district even though Hughenden is outside the target geographic region.

Soils from the surface layer (0-15 cm) of 14 paddocks were collected in September and October 2012 (and a 15$^{th}$ paddock at Warra in southern Queensland was sampled in October 2014). Approximately 50-70 kg of soil was collected from each site and stored at 4$^{o}$ C prior to the commencement of potting. Soils and collection sites are listed in Table 1.

**Table 1: Sample number and locality of soils collected from varying soil-vegetation associations for the glasshouse pot trial to test rhizobia effectiveness in sub-tropical pastures.**

| Sample | Closest town and region | Vegetation/soil description |
|---|---|---|
| 1 | Moura/Bauhinia – central Qld | Brigalow softwood scrub (friable loam) |
| 2 | Moura/Bauhinia – central Qld | Brigalow (grey cracking clay) |
| 3 | Middlemount – central Qld | Brigalow blackbutt (duplex) |
| 4 | Rolleston – central Qld | Basalt downs (black vertosol) |
| 5 | Moura – central Qld | Brigalow belah grey cracking clay (old trial site established with Caatinga stylo) |
| 6 | Tara – southern Qld | Brigalow (grey clay) |
| 7 | Billa Billa – southern Qld | Belah (clay loam) |
| 8 | Talwood – southern Qld | Box sandalwood (hard setting red clay) |
| 9 | Roma – southern Qld | Box (red earth) |
| 10 | Roma – southern Qld | Mitchell grass downs (heavy black clay) |
| 11 | Nindigully – southern Qld | Coolibah floodplain (heavy grey clay) |
| 12 | Manilla – central NSW | Red-Brown chromosol |
| 13 | Breeza – central NSW | Black vertosol (trial 1 only) |
| 14 | Hughenden – north Qld | Cracking clay vertosol |
| 15 | Warra – southern Qld | Coolibah (heavy cracking black vertosol) (trial 2 only) |

**Fig. 1: First glasshouse pot trial just before final harvest date in September 2013.**

The first glasshouse pot trial was established in December 2012 using soils 1 - 14 (Table 1). Treatments were arranged in factorial combination and consisted of:

- Two pasture species (desmanthus (*Desmanthus virgatus* cv. Marc) and Caatinga stylo [*Stylosanthes seabrana* cv. Unica])
- Three rhizobia inoculation treatments: inoculated (using CB3126 for desmanthus and CB3481 for Caatinga stylo); uninoculated; uninoculated + nitrogen (N) fertiliser added)
- Four replicates

This arrangement produced 24 pots per soil type or 336 pots in total. Pots used were approximately 1.5L in volume and were lined with a cloth in the base to prevent soil leakage. Each had its own 3 cm deep saucer. A completely randomised block design was applied. Seeds were inoculated with a peat suspension on the seed just prior to planting. 10 - 12 seeds were sown per pot and emergent seedlings were thinned to three plants per pot 30 days after planting. Basal nutrients (macro and micronutrients) except N were applied to all pots throughout the experiment to eliminate background soil fertility differences and where appropriate N was applied (as one of the three inoculation treatments). Soils most likely had varying N levels at the start of the experiment (note: soils were not analysed for N status) and so a non-limiting supply of N was applied on a weekly basis from 31 January 2013 so as to give 7 mg N per pot per week to one-third of the pots (uninoculated + N treatments). This allowed us to determine whether inoculation could promote plant growth through N fixation to levels comparable to those of plants receiving N.

Shoots were harvested three times (at 3, 8 and 11 months after planting) throughout the experiment and allowed to regrow twice. At the first two harvests, all plants were cut at the fifth node and at the third harvest the entire shoot from soil level was collected. For each harvest the shoots were dried at $60^{o}$ C for 48 hours to determine dry matter (DM) yields. Dry weights for all three harvests were added together to give the total dry weight produced from each pot and expressed on a per plant basis. Prior to the experiment it was determined that a significant increase in dry weight of the inoculated plants over the uninoculated (with the inoculated plants showing equal or trending towards equal response as the +N plants) would be deemed to indicate effective N fixation by the added rhizobia inoculation as compared to any native rhizobia in the nil inoculated treatments.

At the third and final harvest, soil was washed from the roots of all uninoculated pots first. The extraction of roots and assessment of nodules is a lengthy process, and as the main aim was to look at the potential of these species to form nodules in uninoculated soils, it was therefore decided to look at only selected replicates and soils for the inoculated and the uninoculated+N treatments (i.e. those where a significant response in dry weight of the plant tops due to inoculation was measured.). Complete individual nodule counts were not conducted due to the very high numbers of nodules on many of the roots (more than 500 per pot), and so the number of nodules was estimated and then scored on a 0-5 scale. Scores were allocated on the following scale: 0 = 0 nodules, 1 = 1-10 nodules, 2 = 11-50 nodules, 3 = 51-100 nodules, 4 = 101-250 nodules, 5 = 251 or more nodules per pot of three plants.

Following the main experiment, two replicate pots of a selection of the soils (soils 2, 4, 6, 10, 13 and 14) were planted to Caatinga stylo to see if nodulation occurred in the total absence of inoculum. This was a further test of whether native bacteria in some of the soils were capable of forming nodules on Caatinga stylo. All pots received unlimited water and basal nutrients except N. Roots from each of the pots were examined for nodulation after 10 weeks of growth and dry weights of the tops were recorded. Whilst most plants in this extra trial did develop a few small nodules on their roots after two months, there were nowhere near the number that formed on the uninoculated pots in the main experiment. This indicates that there were few if any compatible native rhizobia in those soils and so the uninoculated pots in the main experiment most likely did become contaminated from the inoculated pots.

Due to this suspicion of contamination, a second glasshouse pot trial was conducted in late 2014 using the same soils (collected in 2012) as used for the first trial. All soil had been kept in a cold room at $4^{o}$ C since collection. The only exception was that there was no soil from Breeza, New South Wales, (Soil 13, black vertosol, self-mulching clay) remaining so it was replaced by a black vertosol soil collected from a paddock next to the field trial site at Warra just prior to conducting the second experiment in November 2014 (Soil 15).

All pots and saucers in this second trial were contained in a thick (100 μm) clear plastic bag to eliminate any chance of contamination from one pot to the next due to overwatering and spread of free water. A thin layer of sand (approx. 1 cm deep) was also placed on top of each pot following planting and inoculation, as a means of stopping the spread of rhizobia in water splash or dust movement.

Plants were grown for eight weeks and then harvested. Shoot dry weight, root dry weight, actual number of nodules per plant and weight of nodules per plant were recorded for every pot. Data was analysed in Genstat and was transformed where necessary for analysis of variance. The back-transformed means are presented in this report as they are more meaningful and only the significant interactions are presented graphically.

**Fig. 2: Preparing pots for the second glasshouse pot trial using 14 soils from Queensland and northern New South Wales, November 2014.**

## 3.2 Field trials to compare the relative effectiveness of different rhizobia establishment strategies

Two field sites with different soil types were selected and sown:
The first field site was located at Warra, approximately 130 km west of Toowoomba, Queensland. Soil was a medium to heavy cracking clay. An irrigated and a dryland trial were sown in the same paddock on 20 December 2012. The irrigated section was watered (20 mm) on the day the trial was planted and again (20 mm) at 4, 8 and 14 days after planting. The dryland section received germinating rain 19 days after sowing. Initial germination and plant establishment was low on both sections and so extra seed was broadcast on the plots on 6 March 2013. This and some hard seed breakdown led to an increase in plant numbers in each plot (Fig. 3).

**Fig. 3: View of plots in Warra trial taken at time of sampling in May 2013.**

The second field site was located at Kindon, approximately 150 km south west of Toowoomba, Queensland. This dryland site on a loam soil was planted on 13 March 2013. Sowing was delayed because the site was too wet to plant in December 2012 as planned, then hot and dry conditions prevailed until late summer 2013 when a small fall of rain facilitated sowing in March. Unfortunately, establishment rainfall was low and this resulted in poor and uneven establishment across the plots. A new trial site (Fig. 4) was selected and planting was planned for the summer of 2014. However, the site did not receive sufficient rainfall to plant until 10 February 2015. A germinating rain fell nine days after sowing. This resulted in good plant populations of both species.

**Fig. 4: Field trial at second Kindon site showing plots approximately 3 months after planting.**

At both Warra trial sites and the failed first sowing at Kindon, six rhizobia treatments were compared in four replicated randomised blocks. Treatments were:
1. Control (no inoculant)
2. Control + Nitrogen (no inoculant, N as urea, drilled at 5 cm at 100kgN/ha)
3. Conventional peat covering on seed
4. Seed pellet (commercially coated using peat inoculum)
5. Clay granules (inoculant imbedded within clay, drilled at 5 cm)
6. Water inject (peat inoculum mixed with water, drilled at 5 cm)

At the second sowing at Kindon, six treatments were compared in four randomised blocks. The treatments were:
1. Control (no inoculant)
2. Control + Nitrogen (no inoculant, N as urea, drilled at 5 cm at 100kgN/ha)
3. Conventional freeze-dried inoculant covering on seed
4. Seed pellet (commercially coated using freeze-dried inoculum)
5. Heat-treated millet seed (freeze-dried inoculant coated on the seed, drilled at 5 cm)
6. Water inject (freeze-dried inoculum mixed with water, drilled at 5 cm)

For the Kindon second sowing we were not able to source peat inoculum for either legume so freeze dried inoculum was substituted. We were not able to source clay granular inoculum of either strain of rhizobia for the replanting of the trial at Kindon in February 2015. Instead, sterilised (by oven heating) millet seed was coated with freeze dried inoculum and this was planted at 5 cm depth in the soil in the same way that clay granules would have been.

Species and varieties sown:
- Desmanthus (*Desmanthus virgatus* cv. Marc).
- The main line JCU2 (*Desmanthus virgatus*) in the composite desmanthus cultivar Progardes was sown at Kindon in 2015 as good quality seed of Marc desmanthus was unavailable.
- Caatinga stylo (*Stylosanthes seabrana* cv. Unica)

The seed was drilled into the surface of the soil around 1 cm depth with slight soil coverage followed by a presswheel.

### 3.2.1 Sampling at the Warra field site

Plots of the irrigated trial were sampled for root nodule presence initially in May 2013 to compare nodulation across the inoculation treatments. Five plants per plot were randomly selected and dug up using a garden fork to collect the tap root of each plant and as many lateral roots as possible to a depth of 15 to 20 cm. Plant tops were removed at sampling and soil was then washed from the roots. The number of nodules on the roots were counted. Due to the poor initial establishment of the plants, dry weights of the shoots were not determined as the small number of plants taken was not considered enough to give a true representation of plant growth (high degree of variability).

A second sampling was conducted on 11 April 2014. Each plot from both the irrigated and the rainfed sections was sampled for dry matter production, total N concentration in the shoots and $^{15}N$ natural abundance assays (which measure the amount of N actually fixed by the rhizobia). Roots were also sampled to assess nodulation. The shoots were dried at $60^{o}$C for 48 hours to determine dry matter yields and ground for N analyses. Nodulation was scored by estimating the number of nodules per plant and assigning a rating as per the following 0-5 scale: 0 = 0 nodules, 1 = 1-10 nodules, 2 = 11-50 nodules, 3 = 51-100 nodules, 4 = 101-250 nodules, 5 = 251 or more nodules per sample of five plants.

**Fig. 5: Sampling roots at Warra field site in May 2013.**

### 3.2.2 Sampling at Kindon field site

All plots at the Kindon site were sampled in May 2015 and both plant shoots and roots were collected. Shoots were dried at $60^{o}$ C for 48 hours and weighed. Soil was washed carefully from roots and assessed for nodulation by counting the number of nodules present. This method differs from the way the nodules were assessed at Warra because it was obvious when the soil was washed from the roots that there were very few nodules in the samples. Roots were then dried and weighed.

**Fig. 6: Sampling shoots and roots at Kindon field trial (desmanthus treatment).**

# 4 Results

## 4.1 Glasshouse trials to evaluate the effectiveness of native rhizobia on Caatinga stylo and desmanthus

### 4.1.1 Glasshouse pot trial number 1

Nodule assessments
In this experiment, the majority of the roots from the uninoculated and uninoculated + N pots for both species of legumes were found to be well nodulated (scored 3, 4 or 5, i.e. more than 50 nodules per pot of 3 plants) and there was no difference from the inoculated plants. Therefore, it was concluded that either a high level of contamination had occurred in the uninoculated treatments, or that the soils tested contained high numbers of native rhizobium which had nodulated the host plant in the absence of the commercial rhizobium strains.

Analysis of variance of the root nodulation scores for just the uninoculated treatments showed significant differences between the soil types and between the two legume species but no interactive effects. Both species averaged a nodulation score of above 3 (more than 50 nodules per pot) (Fig. 7), with the exception of soil 6 (grey clay from the Tara region in southern Queensland) and soil 12 (red-brown chromosol from near Manilla, New South Wales). On average, the desmanthus plants formed more nodules than the Caatingo stylo plants.

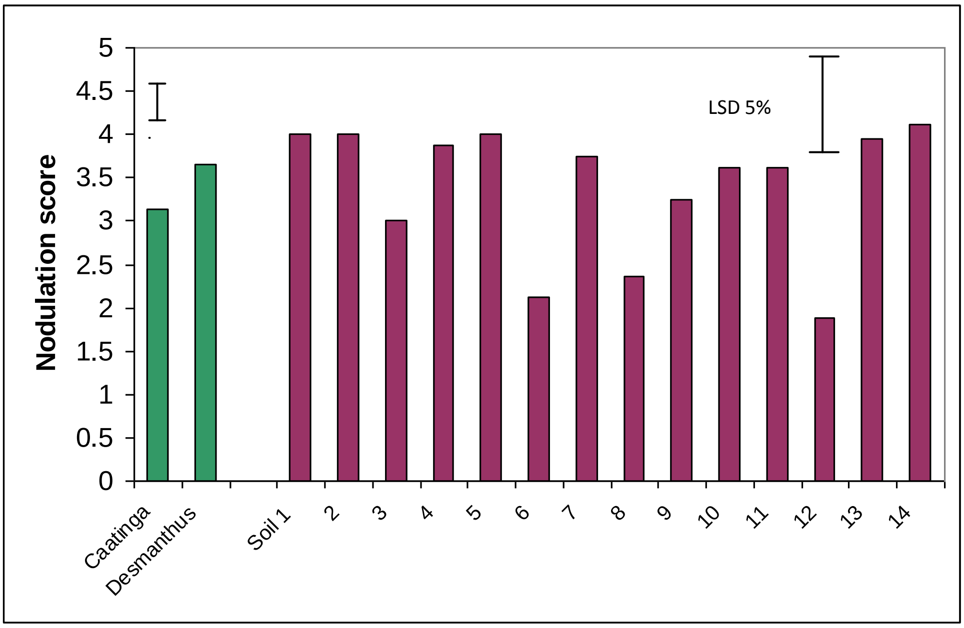


**Fig. 7: Nodulation of uninoculated pots for the two legume species grown in glasshouse pot trial 1. Caatinga stylo (*Stylosanthes seabrana* cv.Unica) and desmanthus (*Desmanthus virgatus* cv. Marc), averaged across all soils (green bars). Purple bars show scores for each soil averaged across both species. There was no interaction between legume species and soil. Scores were allocated on the following scale (approximate numbers) 0 = 0 nodules, 1 = 1-10 nodules, 2 = 11-50 nodules, 3 = 51-100 nodules, 4 = 101-250 nodules, 5 = 251 or more nodules per pot of three plants. (Refer to Table 1 for key of soil numbers)**

Plant growth responses

Caatinga stylo showed significant dry weight responses to inoculation in only two of the 14 soils tested (Fig. 8). These were soil 6 (a brigalow grey clay from Tara, southern Queensland) and soil 8 (a box sandalwood red clay from Talwood, southern Queensland). Soils 2 (grey clay from Moura, central Queensland), 3 (duplex soil from Middlemount, central Queensland) and 11 (a grey clay from Nindigully, southern Queensland) also showed small growth improvements with the application of inocculant though they were not significant. These results are confounded by the suspected contamination of uninoculated pots.

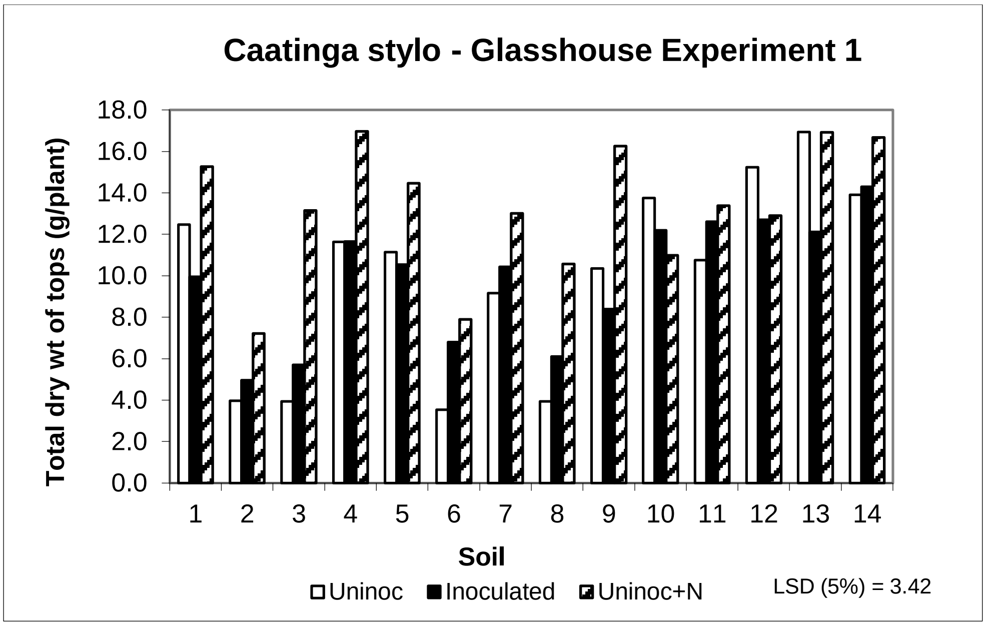


**Fig. 8: Pot trial growth response of Caatinga stylo (*Stylosanthes seabrana* cv. Unica) to rhizobial inoculation or to applied nitrogen. Pots were grown for 10 months in 14 different soils collected from grass pastures across Queensland and New South Wales.**

Analysis of the total dry weight of plant tops data for the desmanthus plants showed that soils and inoculum treatments had significant effects but there was no interaction of these two factors. The results for the full set of treatments are shown in Fig. 9. Soils 2, 6, 8, 9 and 12 produced low total dry weights even when inoculated. There was no significant response to inoculation (confounded by possibility of contamination in the uninoculated pots), only to applied N. For most soils there was no way of measuring whether the nodules were functioning effectively to fix N as there were no growth differences between inoculated and uninoculated plants. For this reason, the cost of nitrogen analyses of the tops was not considered to be warranted.

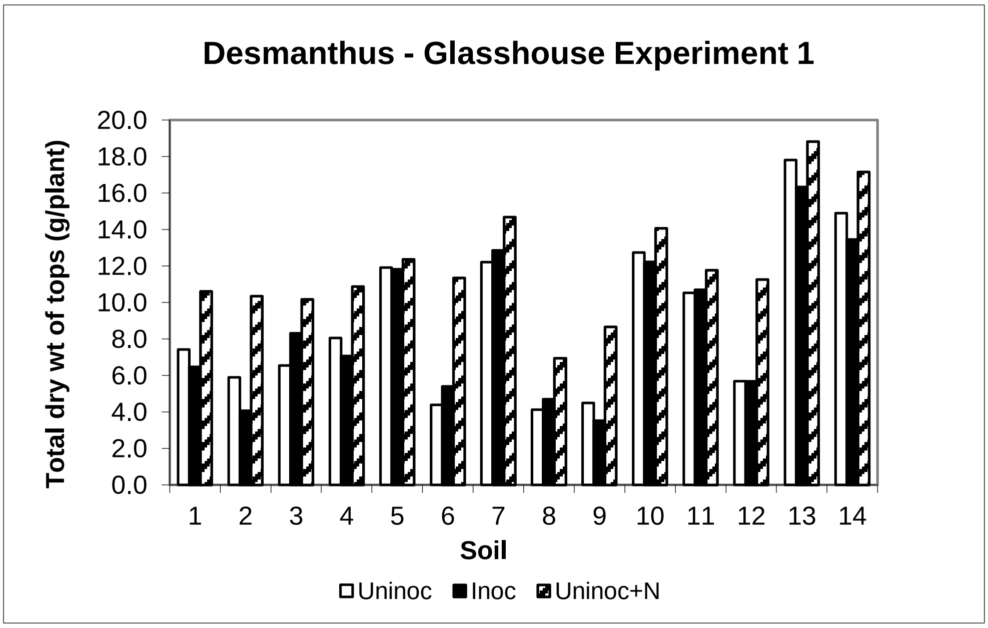


**Fig. 9: Pot trial growth response of desmanthus (*Desmanthus virgatus* cv. Marc) to rhizobial inoculation or to applied nitrogen. Pots were grown for 10 months in 14 different soils collected from grass pastures across Queensland and New South Wales.**

For both plant species, the main significant differences in inoculum treatments for each of the three individual harvests were in response to the applied N, indicating that the soils were generally low in N fertility and that inoculation was unable to amend this shortfall with fixed atmospheric N. With these very limited responses to inoculation and the suspected contamination of uninocualted pots, it was decided that the experiment required repeating.

### 4.1.2 Glasshouse pot trial number 2

Nodulation assessments

The number of nodules per plant was significantly affected by the interaction of pasture species, soil and inoculation treatment. The number of nodules on the Caatinga stylo plants was generally higher than in the desmanthus plants for most of the soils.

There were more nodules on the inoculated than the uninoculated pots of Caatinga stylo except in soils number 5 (a soil from an established Caatinga stylo paddock near Moura, central Queensland where some of the compatible rhizobia may have survived) and number 15 (black Vertosol from Warra, southern Queensland). Although not significant there were also more nodules on the inoculated than the uninoculated from soil number 9 (red earth from Roma, southern Queensland), number 10 (black Vertosol from Roma, southern Queensland), number 14 (Vertosol from Hughenden, northern Queensland (Fig. 10).

Nodulation on desmanthus was low, even with inoculation, in many of the soils except for soils number 4 (black Vertosol from Rolleston, central Queensland), number 14 (Vertosol from Hughendon, selected because the native legume neptunia (*Neptunia gracilis*) was growing and is suspected as being a host to a rhizobia compatible with desmanthus) and number 15 (black Vertosol from Warra). Whilst the uninoculated plants in these three soils also produced some nodules, the inoculated plants produced significantly more (Fig. 11).

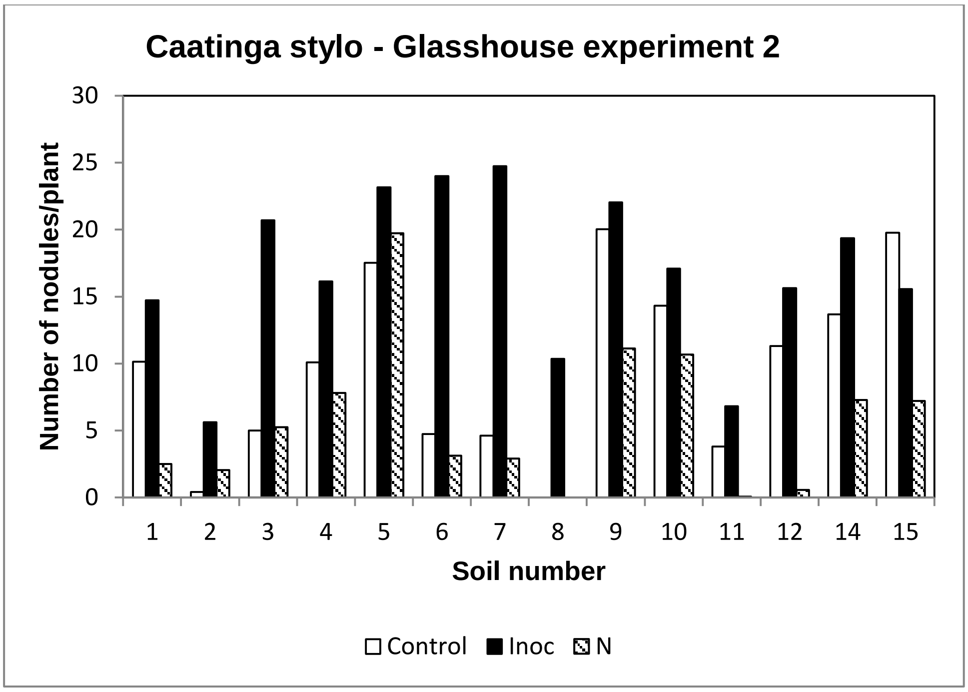


**Fig. 10: Average number of nodules per plant (back-transformed data) on roots of Caatinga stylo grown in each of the soils sampled from grass-only pastures from across Queensland and northern New South Wales.**

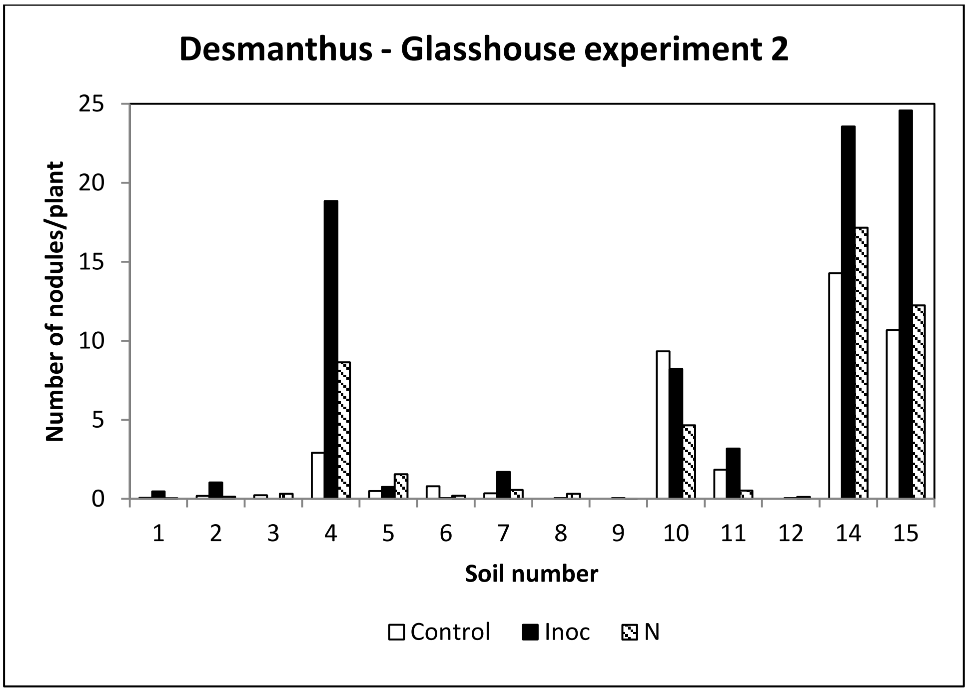


**Fig. 11: Average number of nodules per plant (back transformed data) on roots of desmanthus grown in soils sampled from grass-only pastures from across Queensland and northern New South Wales.**

<u>Plant growth responses</u>

Statistical analysis of the dry weight responses showed a significant interaction of inoculation treatment x legume species (averaged across all soils) The Caatinga stylo in general responded with better growth when inoculated (equal to that when provided with N fertiliser) on average across the soils (Fig. 12). However, desmanthus plants did not respond to inoculation. When N fertiliser was applied, growth of both Caatinga stylo and desmanthus was the same (Fig. 12).

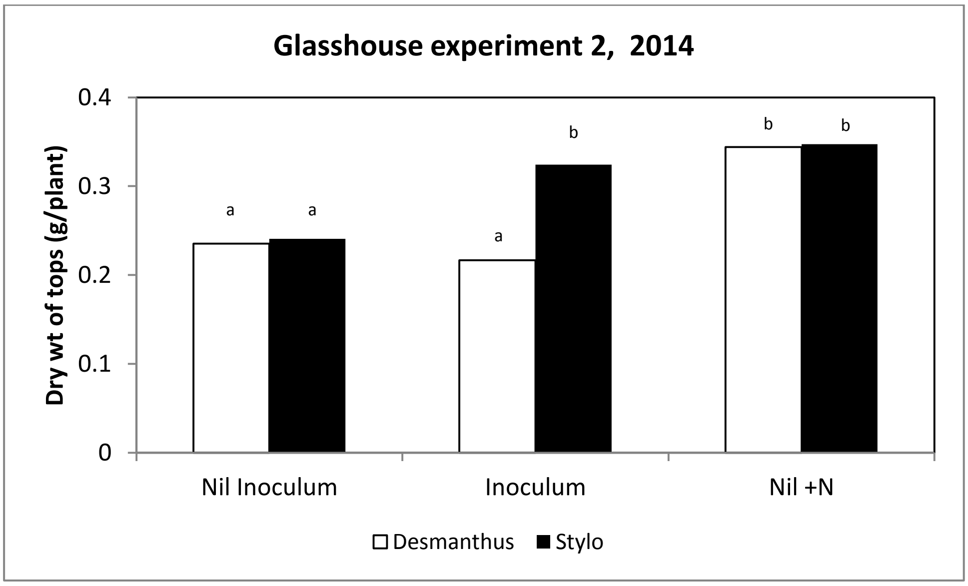


**Fig. 12: Dry matter response (back-transformed data following analysis of variance of ln [drywt + 0.1]) of desmanthus and Caatinga stylo to the addition of inoculum or N fertiliser averaged over 14 different grass pasture soils from across Queensland and northern New South Wales.**

Although the three-way interaction of pasture species x soil x inoculum treatment was not statistically significant, it is more meaningful to look at the responses for individual species at each soil type and inoculum treatment. These are given in Figures 13 and 14 below. For Caatinga stylo, responses to inoculation were generally positive, particularly in soil 5 (even though there was a background of Caatinga stylo having been successfully grown in this soil for 15 years and there were high levels of nodulation even in the uninoculated pots). Soil 15 (from Warra, southern Queensland) also showed a good response to inoculation despite there being many nodules present in the uninoculated pots. The desmanthus plants show that across all soils there was no response to inoculation even though there were significantly more nodules present on inoculated than uninoculated plants in some soils (soils 4, 14 and 15 in particular).

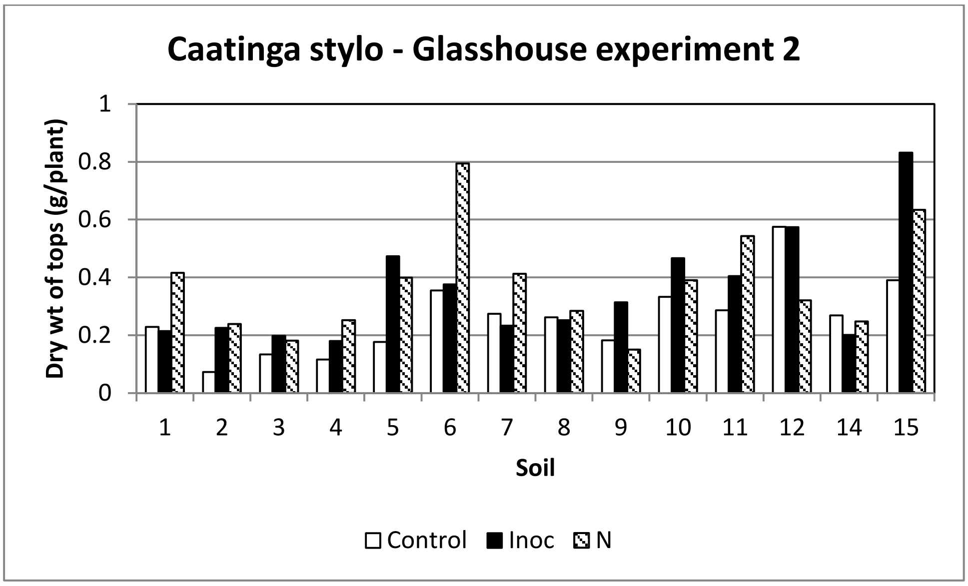


**Fig. 13: Dry matter response (back-transformed data following analysis of variance of ln [drywt + 0.1]) of Caatinga stylo after 56 days growing in 14 different grass-only pasture soils from across Queensland and northern New South Wales for the nil, inoculum, and N fertiliser treatments**

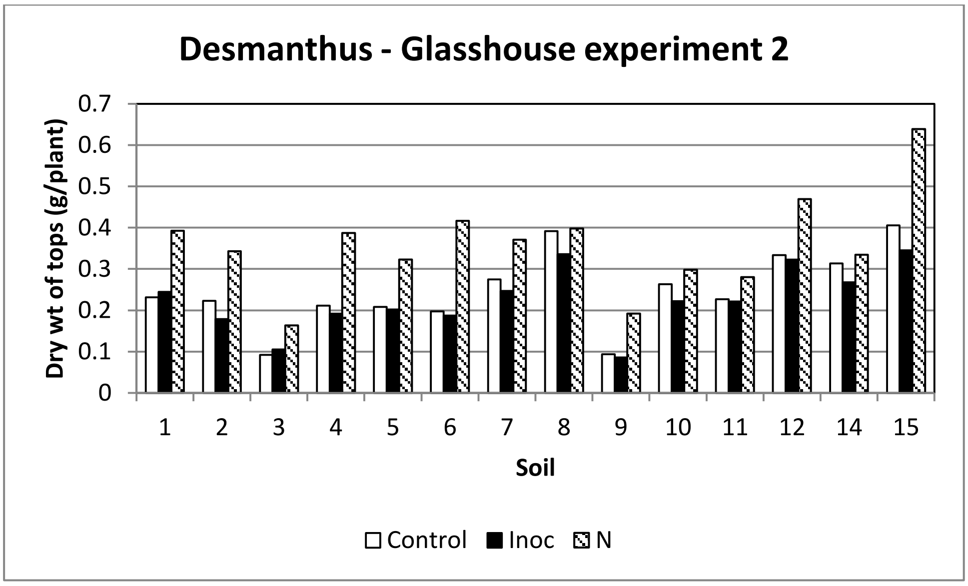


**Fig. 14: Dry matter response (back-transformed data following analysis of variance of ln [drywt + 0.1]) of desmanthus after 56 days growing in 14 different grass-only pasture soils from across Queensland and northern New South Wales for the nil, inoculum, and N fertiliser treatments.**

## 4.2 Field trials to compare the relative effectiveness of different rhizobia establishment strategies

### 4.2.1 Warra field trial, southern Queensland

Nodulation assessments
Results of the root nodule sampling carried out on the irrigated section of the trial in May 2013 are presented in Fig. 15.

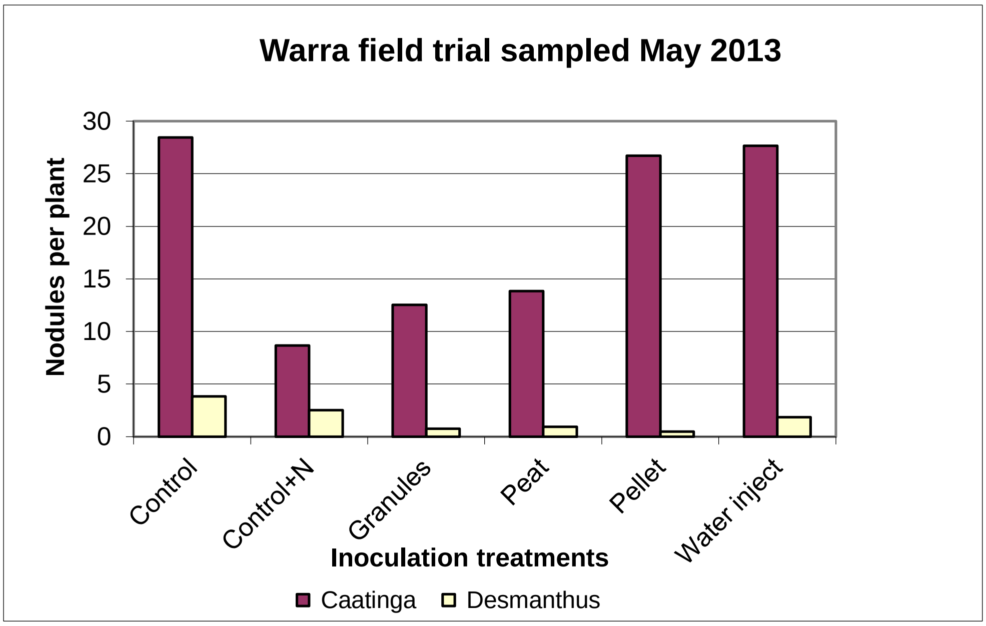


**Fig. 15: Number of nodules per plant for the different rhizobia treatments applied to Caatinga stylo and desmanthus within the irrigated section at Warra (LSD 5% = 8.76).**

The results from the first sampling (May 2013, five months after planting) recorded more nodules on Caatinga stylo plants compared to desmanthus (Fig.15). Caatinga stylo produces many small nodules as opposed to desmanthus that produces fewer nodules that are larger. Desmanthus nodule counts were however very low in general and not significantly different between the treatments.

The Caatinga stylo control plots (no inoculum) appeared to have a relatively high number of nodules compared to the other treatments. These nodules were not evaluated for effectiveness or weight. However the Caatinga stylo control + N plots had a much lower number, indicating that applying nitrogen may have reduced nodulation significantly. It is probable that the nodules have been produced by infection by native rhizobia. However, without reliable data on production (dry weights of the plant tops) to relate this to, it cannot be established with certainty whether these nodules were effectively fixing N for the plant and hence influencing biomass production.

The pelleted Caatinga stylo seed produced just as many nodules as the water injection treatment however, with the control treatment producing as many nodules as it did, it is difficult to make judgements on the effectiveness of the inoculum.

Initial soil fertility analysis showed the site had very low nitrate nitrogen in the profile (6 kg/ha in the 0-120 cm of soil). In such low N fertility conditions a dry matter response to inoculation would usually be expected as a result of rhizobial N fixatuion. Some mineralisation of N would have occurred throughout the trial which would also have contributed to legume N nutrition. Phosphorus and zinc levels appeared adequate in the top 10 cm layer of soil but low below that depth. At this site pH was high, ranging from 8.4 in the surface 0-10 cm to 9.0 below 10 cm.

A second sampling for nodulation and dry matter production was carried out at this site in April 2014 (16 months after planting). Both the irrigated and rainfed trials produced low but comparable levels of plant nodulation. Caatinga stylo had a significantly higher overall score than the desmanthus (respective scores of 2 and 1) in both trials. In the irrigated section, nodulation in the Caatinga control plots was just as good as all inoculated plots however nodulation was less (not significantly) in the Control+N plots. For desmanthus, low numbers were recorded across all treatments with the highest number being observed on the control (uninoculated), followed by the Control+N and water inject, with the peat, pellet and granule treatments producing negligible nodule counts (Fig.15). These observed differences were however not significant. It was therefore difficult to discern a major impact of inoculation from these results as the control (uninoculated) treatments were as well nodulated as other inoculated treatments.

## Dry matter production

Caatinga stylo consistently produced more shoot biomass in all treatments than did desmanthus in both the irrigated and rainfed trials (Figures 16 and 17). Production was generally higher in the irrigated than the rainfed trial as expected, due to more favourable soil moisture conditions. Whilst significant differences between the inoculum treatments did not occur, there was an observed tendency toward a dry weight response in Caatinga stylo to the addition of N and water injected inoculum especially in the irrigated trial and to the use of granules in both trials (Figures 16 and 17).

There were low plant numbers remaining from the initial establishment which meant that sampling was limited to only five plants per plot instead of the 15-20 plants that would have been preferable. This may have contributed additional variability to the data and hence the calculation of less significant differences between treatments even though some trends were evident.

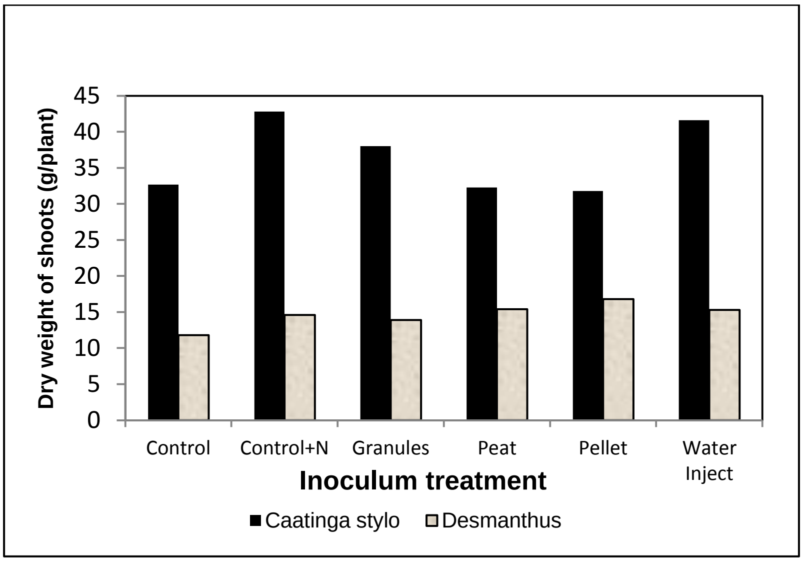


**Fig. 16: Dry weight of shoots of the desmanthus and Caatinga stylo 16 months after planting in the irrigated field trial at Warra, southern Queensland in response to different methods of rhizobia inoculation at planting (Note: the only statistically significant difference was that between the legume species grown).**

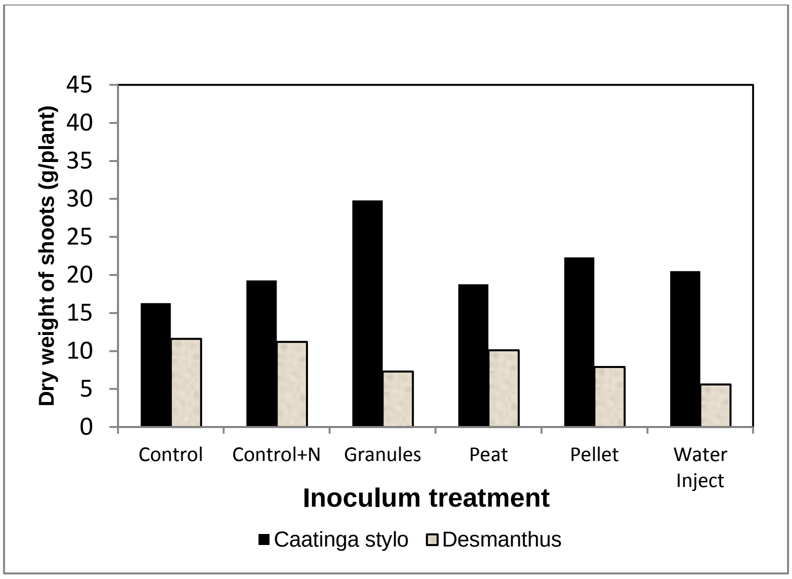


**Fig. 17: Dry weight of shoots of the desmanthus and Caatinga stylo 16 months after planting in the rainfed field trial at Warra, southern Queensland in response to different methods of rhizobia inoculation at planting (Note: the only statistically significant difference was that between the legume species grown).**

<u>Nitrogen concentration and content of shoots</u>

The results for the concentration of N in the shoots are presented below in (Figures 18 and 19). In the irrigated section, a significant legume x inoculum treatment interaction (LSD (5%) = 0.293) occurred with the Control+N and the water injection inoculum treatments in desmanthus being significantly higher in N concentration than the control and the pelleted seed. Otherwise N% was not significantly influenced by inoculum treatment.

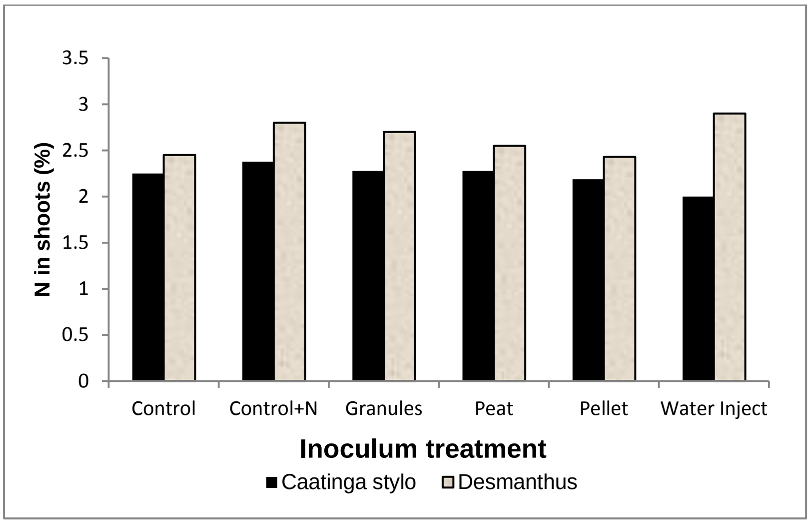


**Fig. 18: Nitrogen concentration (%) in the shoots of the desmanthus and Caatinga stylo 16 months after planting in response to different methods of rhizobia inoculation at planting in the irrigated field trial at Warra, Queensland (LSD (5%) = 0.293).**

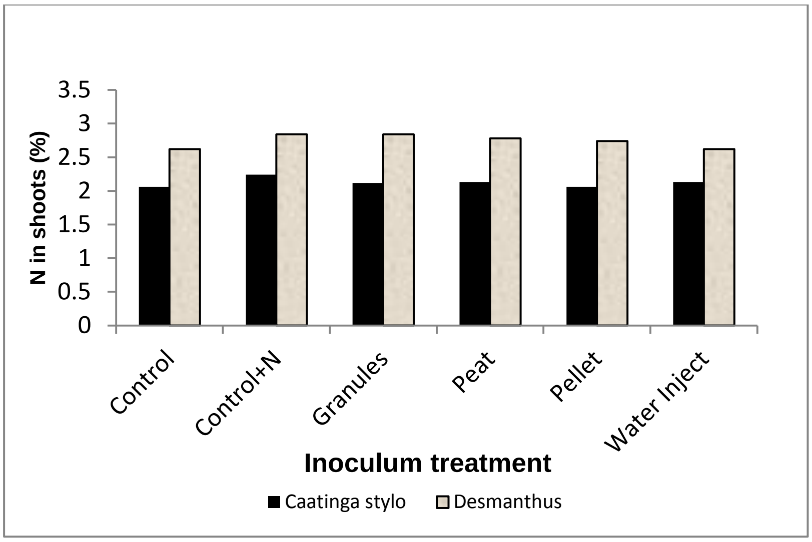


**Fig. 19: Nitrogen concentration (%) in the shoots of the desmanthus and Caatinga stylo 16 months after planting in the rainfed field trial in response to different methods of rhizobia inoculation at planting at Warra, Queensland.**

The total amount of N in the shoots (N concentration x dry weight of shoots) is given in Figures 20 and 21. The Caatinga stylo plants showed a trend (not statistically significant) of having more N in the tops in the Control+N treatment and also the granules (irrigated and rainfed trials) and water inject (irrigated only) treatments, with the peat and pelleted treatments demonstrating seemingly lower N uptakes. The extra N in the shoots is primarily due to the higher biomass produced by these treatments, not due to a higher N % concentration.

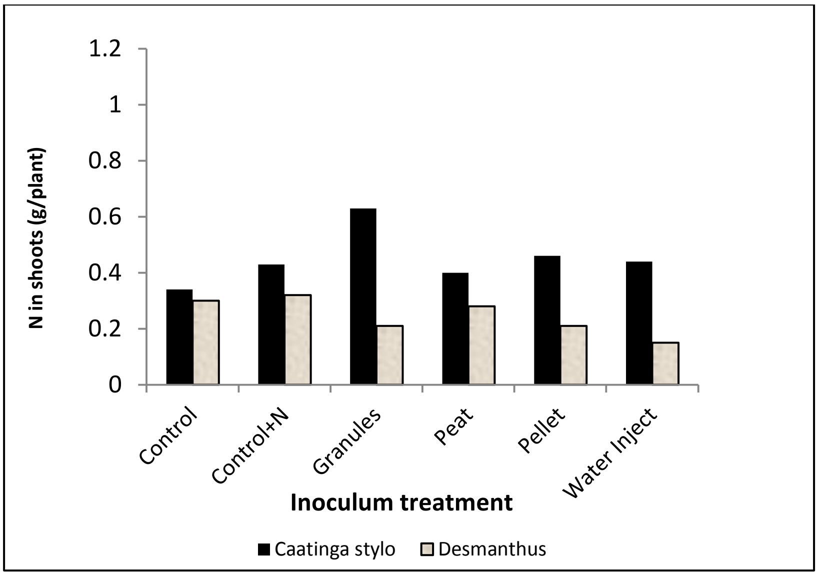


**Fig. 20: Nitrogen content in the shoots of the desmanthus and Caatinga stylo 16 months after planting in the irrigated field trial in response to different methods of rhizobia inoculation at planting at Warra, southern Queensland. (Note: the only statistically significant difference within each trial was that between the legume species grown).**

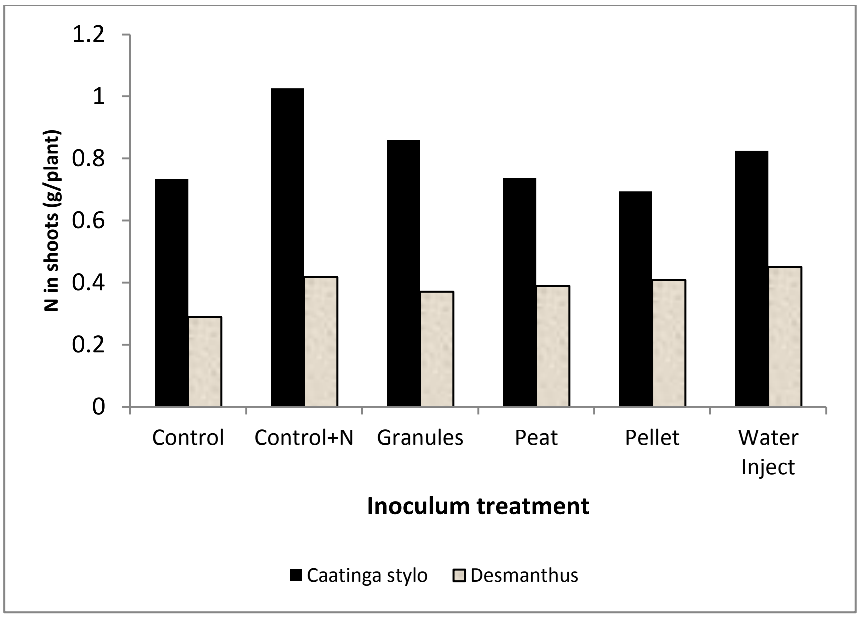


**Fig. 21: Nitrogen content in the shoots of the desmanthus and Caatinga stylo 16 months after planting in the rainfed field trials in response to different methods of rhizobia inoculation at planting at Warra, southern Queensland. (Note: the only statistically significant difference within each trial was that between the legume species grown).**

<u>Nitrogen fixation</u>

The nitrogen concentration results presented so far are a combination of N taken up from both the soil and from fixation by rhizobia on the roots. As well as total N in the shoots, the $^{15}N$ content of shoots was analysed. From this it was possible to calculate the proportion of that N that was biologically fixed from the atmosphere (% Nitrogen derived from the atmosphere or %Ndfa). These are presented in Fig. 22 for the Caatinga stylo plots only. Difficulties with getting a suitable B-value (needed to calculate the %Ndfa) for desmanthus plants and some very low $^{15}N$ values gave rise to nonsensical results for this species. Further experimentation, data analysis and investigation is required to clarify these results. Considering the Caatinga stylo results, the %Ndfa was low, averaging around only 20% or less and the two trials gave extremely different results in their $^{15}N$ profiles, although the corresponding nodule scores did not reflect this difference. Some similarities to the dry weight responses can be seen in the %Ndfa results, for example the granules treatment in the rainfed trial had higher N and higher dry weight as well as a significantly higher proportion of the N in the tops coming from the fixed N. Water injection was the only other treatment to significantly increase N fixation in the rainfed trial but again only 5% of the N in the tops was estimated to have come from the atmosphere (i.e. via fixation). However, in the irrigated trial the granules treatment had the lowest %Ndfa (significantly lower than the controls, pelleted and water inject treatments) and inoculation seems to have had no effect on N fixation whatsoever.

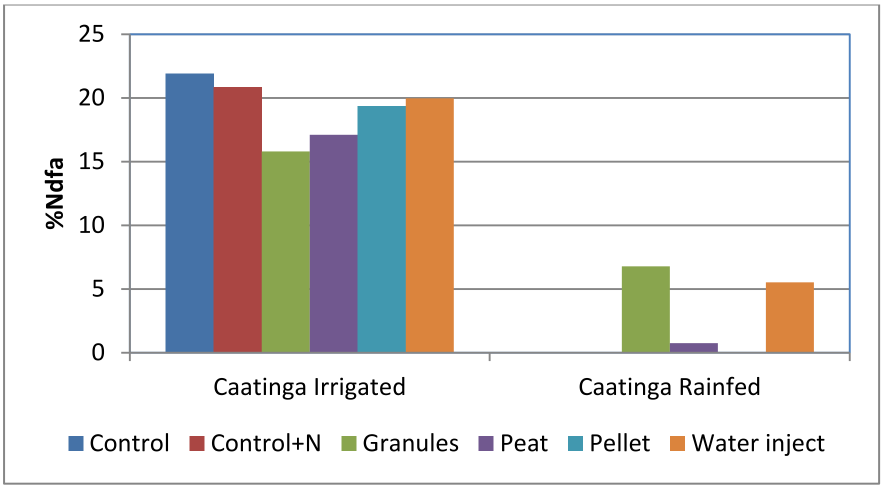


**Fig. 22: The mean percentage of nitrogen in the plant tops that was derived from the atmosphere via N fixation (%Ndfa) for the Caatinga stylo plants in the irrigated and rainfed sections of the Warra trial site, southern Queensland in April 2014 (Note. Analysis of variance of the two trials separately showed no significant differences between the treatments due to highly variable results).**

### 4.2.2 Kindon field trial, southern Queensland

At the time of sampling in April 2015 (about three months after planting), the dry weights of the plant tops at Kindon revealed no significant differences in plant growth in response to the addition of inoculum. Root nodulation was not detected apart from a few nodules on random treatments. An analysis of the soil showed high levels of soil nitrate in the profile and this is likely to have been the reason for the lack of nodulation. A crop of barley has been sown over the entire site with the aim of reducing nitrate levels. When the legume pastures rejuvenate next summer, all treatments will be resampled for the measurement of nodulation, shoot growth and N fixation differences.

# 5 Discussion

## 5.1 Glasshouse trials to evaluate the effectiveness of native rhizobia on Caatinga stylo and desmanthus

In the first pot experiment, results showed that across a range of 14 soils investigated only a very limited number of soils (numbers 6 and 8, the grey and red clays from southern Queensland) improved growth with inoculation of the two legumes. However, very few of the uninoculated pots had nodulation levels significantly lower than the inoculated pots. Whilst some nodulation, particularly on the desmanthus roots, may have been expected due to native rhizobia in the soil (Bahnish 1998), the amount of nodulation suggests that there was most likely contamination with rhizobia from inoculated pots. Caatinga stylo has been shown to have a very specific strain compatibility for effective nodulation (Date 2010) and yet plants that were not inoculated were well nodulated. Whilst it cannot be proven that the nodulation was not caused from native bacteria without extensive molecular tests being carried out, it is probable that contamination occurred as the pots were initially positioned very close together in a small glasshouse room and rhizobium transfer via air movement and water splash could have occurred. Also, the experiment ran for several months looking for evidence of a dry weight response, and thus allowed more time for contamination and nodulation to take place.

The glasshouse experiment carried out in 2014 (Experiment 2) showed that in general across all soils, the addition of the commercial strain of rhizobia CB3481 greatly improved growth of Caatinga stylo as much as additional N did, even in just eight weeks of growth. This indicates that the strain CB3481 is very effective at fixing N in this legume species and that inoculation is vital for the productive growth of Caatinga stylo.

The addition of the desmanthus commercial strain (CB3126) did not significantly improve plant growth in any of the soils, however, adding N did. Nodulation was increased due to inoculation in only three soils (4, 14 and 15) which interestingly all happen to be from similar soil types (vertosols). However, this high nodulation due to inoculation with CB3126 did not translate into better plant growth than any of the uninoculated plants. In some of the soils tested, uninoculated plants grew just as well as the plants that were given N fertiliser (soils 8, 10 and 14). Bahnisch et al. (1998) concluded that six out of eight soils that they screened for response to inoculum in desmanthus had indigenous strains of rhizobia with capacity to nodulate (most of which were as effective as CB3126, the commercial inoculant strain). Desmanthus plants grown in soil number 14 nodulated well without inoculum also. This soil was collected from a site where the native legume neptunia (*Neptunia gracilis*), thought to be a host for a rhizobia strain that infects desmanthus, was growing (Date 1991). Bahnish et al. (1998) did however still find four soils where nodulation by indigenous strains was poor and inoculation with CB3126 increased plant growth. Our study results seem to indicate that CB3126 has no advantage in DM production over the indigenous strains in any of the soils screened despite soils having more nodules, however with no easy way for growers to tell if their soil has indigenous strains or not, inoculation is still recommended for desmanthus.

## 5.2 Field trials to compare the relative effectiveness of different rhizobia establishment strategies

In the field trial site at Warra, southern Queensland, Caatinga stylo was generally better nodulated than desmanthus across all treatments including controls and had higher dry weight production as well as higher amounts of N in the shoots regardless of soil moisture conditions. In Caatinga stylo, there was a trend for higher dry weights and N concentrations in the plants inoculated with granules or via water injection, and this correlated with a higher proportion of the N measured in those treatments being atmospherically derived due to fixation than in the other treatments (albeit a very small proportion of less than 20% in the irrigated and less that 5% in the rainfed sections). This could be a reflection of these treatments being applied at depth and thereby affording some protection of the rhizobia compared to the seed coated with peat or the pelleted seed which were mainly on the soil surface. Previous research (Brockwell et al. 1980, Denton et al. 2009) found that granular formulations used in Australian legume crops produced equivalent nodulation and yields in legumes to those achieved via seed-applied peat inoculants, provided inoculant bacterial counts were high. Granular inoculants were also found to be effective at providing greater nodule numbers and nodule mass than peat slurry on lucerne (Rice and Olsen 1992). Denton et al. (2009) also stated that granular inoculants may have a role in providing an effective inoculant in dry sowing conditions, which could be very applicable to pasture systems in the tropics.

The second glasshouse experiment gave some useful insights into the results obtained from the field trial at Warra, particularly for Caatinga stylo. The control (uninoculated treatments) in both the field and the glasshouse trials in the Warra soil had a high number of nodules on the roots indicating a competitive native strain was able to form nodules on the Caatinga stylo. Whilst growth of the plants was not improved over the inoculated treatments, the field trial showed just as much N was being fixed in the irrigated section control plots as in other treatments. Further investigation of this native strain of rhizobia in this Warra soil is warranted.

The project faced many challenges during both the glasshouse experiments and the field trials. Firstly it was decided to extend the growing time of the legumes in the first pot trial in an attempt to determine growth differences between treatments and the different soils. This most probably led to increased contamination potential between treatments. The second pot trial was run for a much shorter time frame to reduce contamination but could have been at the detriment of measuring larger differences in production and nodulation between the treatments.

The field trials also faced issues that were out of the control of project staff. Extremely high temperatures at Warra following establishment irrigation and not being able to follow with the second watering quickly (because of the very wet soil just under the surface) allowed the surface soil (where the seed was placed) to dry out more rapidly than expected, reducing germination and establishment. The Kindon site experienced extremes of wet and dry. Firstly it was too wet to plant at the preferred time, and then following planting, limited rainfall was received reducing plant populations. The first site was abandoned and a new site selected which was to be sown the next summer. This proved to be an exceptionally dry period and no sowing could be done which meant that the site was long fallowed, allowing nitrogen to accumulate at the site. Due to the high level of available N, following sowing, no nodulation was detected as the plants were using the large amounts of available soil N.

## 5.3 Meeting objectives

The objectives of this project were met in the following ways:

1. **Evaluated the potential of native rhizobia from grass-only pastures/soils in central Queensland, southern Queensland and northern New South Wales to form effective nodules on key legume species (including Caatinga stylo and desmanthus) that are currently considered to have specific rhizobia requirements.**
   Results from pot trial studies showed that whilst some soils contained bacteria capable of nodulating desmanthus and Caatinga stylo, the inoculum treatments using commercial strains of rhizobia improved nodulation and growth in some of the soils in particular for Caatinga stylo. This concludes that these legumes should always be inoculated with commercial inoculant strain when sowing in commercial pastures.

2. **Compared the relative effectiveness of different rhizobia establishment strategies in difficult conditions (i.e. small seeds, hot and dry summer) that are common when establishing sub-tropical pastures.**
   The results from the field trials, although not statistically significant, indicate the potential for improved dry weights and nitrogen contents from the application of commercial inoculum via granules or water injection. This is more evident for Caatinga stylo in the rain fed trial where nodulation increased with the clay granules and water injection treatments both of which place the inoculum at depth.

3. **Produced guidelines and specific recommendations to establish rhizobia with legumes in tropical and sub-tropical pastures.**
   The outcomes of this project were not as definitive as would have been expected. There are indications that increases in rhizobia effectiveness can be achieved by placing the inoculum at depth in the soil. This information will be relayed to producers attending workshops conducted within the “Improving productivity of rundown sown grass pastures” project (B.NBP.0639). Information will be relayed to staff in New South Wales working on tropical legumes following the final sampling at the Kindon site in 2016.

# 6 Conclusions/recommendations

## 6.1 Key insights

Introducing legumes into grass pastures is seen as the most economical long term cost-effective method of reducing productivity decline in sown pastures. Improving effective nodulation of these introduced legumes will increase the nitrogen content of the legumes which improves feed quality to livestock and increases the dry matter production of the legumes thus increasing soil nitrogen contribution to the associated grasses. The results from this study have led the project team to the following conclusions:

1. **Caatinga stylo should always be inoculated**
   The recommendation that Caatinga stylo should always be inoculated when planting the legume into/with grass pastures came from previous studies where it was found to be very specific in its bacterial requirements and few, if any soils could supply the bacteria required for nodulation.

   The pot trial in this study found that inoculation of Caatinga stylo increased plant nodulation on around 95% of the soils tested and increased dry matter production on around 50% of those soils. This data supported the findings presented in a paper by Date (in preparation, 2015) in which Caatinga stylo failed to form nodules within 13 soils collected from across Queensland

2. **The vertosol soil at the Warra site may have potential to form effective nodules on Caatinga stylo**
   Despite previous studies finding Caatinga stylo does not form effective nodules with native bacteria, it appears that nodulation has occurred with the soil collected from the Warra trial site in southern Queensland. In the second pot trial and the control treatments in the Warra irrigated field trial there were as many if not more nodules in the control as other inoculated treatments, however DM was improved with inoculation in the pot trial.

3. **Deeper placement can increase inoculum survival compared with shallow placement**
   Surface planting small seeded summer growing legumes into dry hot soil and waiting for follow up rain for germination can be detrimental to the survival of the inoculant applied on the seed coat. When planting legumes including Caatinga stylo and desmanthus where an establishment rain is unlikely to occur within quick succession of sowing, applying the inoculum below the surface (5 cm) can improve nodulation and N fixation. This was shown in the dryland site at Warra where both clay granules and water injection of inoculum improved both the dry matter production and the Ndfa %. This is supported by Date's (2015) studies where the author recorded more nodules on Caatinga stylo from inoculum coated on either wheat seed or plastic balls placed at depth in the soil.

4. **Desmanthus should be inoculated**
   There are some advisors suggesting there is no need to inoculate desmanthus as it will form nodules with native bacteria. Although the results from this project and previous work suggest that desmanthus can form nodules with some native bacteria, there is no evidence to support improved dry matter production when these nodules are formed nor is there a practical, cost effective way of testing which soils require inoculating. Therefore, it is recommended that desmanthus be inoculated. In this study, the inoculation of desmanthus increased nodulation in about 50% of the soils tested.This was shown in the second pot trial in which 8 out of the 14 soils tested increased nodulation but failed to increase dry matter production.

5. **When sowing seed into hot, dry soil where rain or irrigation does not immediately follow, survival of inoculum is reduced**
   Both of the small seeded summer growing legumes investigated are limited in the depth at which they can be sown (maximum 20 mm for desmanthus, 10 mm for Caatinga) for reliable establishment and therefore must be planted either shallowly or broadcast on the soil surface. This limits the ability to place seed onto soil moisture and means that seed-applied inocculum can be exposed to dry soil where temperatures can be too hot for rhizobia to survive. This risk can be overcome by placing inoculum at greater depths (eg. 5 cm) via water injection or granular application. The Warra irrigated trial demonstrated that if rain falls or irrigation is applied soon after sowing, the application of inoculum at the surface or subsurface of the soil results in similar rhizobial survival. In this experiment, all inoculum treatments nodulated and fixed nitrogen from the atmosphere as measured in the Ndfa results for all inoculated treatments of both Caatinga stylo and desmanthus. In the dryland site the Caatinga stylo plants inoculated with the clay granules and water inject were able to fix more nitrogen from the atmosphere suggesting nodulation was increased under those treatments (however this was not significant). In cases where water injection or granular placement of inoculum to depth is not feasible, it is recommended that sowing be planned for a period in which rainfall is expected and high soil surface temperatures are unlikely.

6. **Legumes fix nitrogen when soil N is low**
   Legumes don't nodulate and fix nitrogen from the atmosphere when there is an ample supply of available N in the soil. This was proven in the results from the sampling of the Kindon field site which had been long fallowed and had accumulated above 150 kg of available nitrogen/ha.

The project team are committed to continuing the Kindon field site and sampling for nodule numbers in the next growing season. A crop of barley has been sown over the plots and the biomass will be cut and removed in an attempt to reduce the amount of available nitrogen for the legumes to use. When the legumes are in peak production next summer, the treatments will be sampled for nodulation and shoot growth differences. Assuming the bacteria have survived this should then provide insights into the applied treatments. The team needs to negotiate how the results will be reported to MLA.

This project showed that inoculating with commercially available specific strains of rhizobia improved inoculation of Caatinga stylo in particular and that the best methods to do this practically are by placing the inoculum deeper in the profile than the seed via either water injection or granular formulations. This supports the findings of previous studies on Caatinga stylo suggesting the species is less likely to effectively nodulate with native bacteria.

## 6.2 Future RD&E

The results from this project suggest that there can be improvements made in increasing the effectiveness of different methods of applying rhizobia at sowing. It also highlighted a number of issues requiring further investigation as RD&E priorities:

- Effectiveness of deeper placement of inoculum
- Determination of inoculum survival in storage
- Provision of information to industry on inoculum requirements
- Determination of neptunia as a host for compatible rhizobia for desmanthus
- Determination of the capacity of the Warra soil to nodulate Caatinga stylo effectively

- Effectiveness of alternative host legumes as carriers of inoculum

**Effectiveness of deeper placement of inoculum**
Cropping systems are using water injection or clay granules as convenient, time efficient and reliable methods of inoculating crop legumes. Both of these newer techniques can deliver the inoculum deeper into the soil where temperatures are lower and can be applied when the soil is moist which should improve inoculum survival.

Although this study didn't prove this conclusively, there is enough evidence to suggest there is a requirement to demonstrate that when sowing summer growing pasture legumes, by placing the inoculum in the sub-surface soil, survival is greater and should lead to increased nodulation which will increase dry matter production and nitrogen fixation.

If clay granules are shown to be the best method of introducing the inoculum, the challenge of convincing commercial inoculum manufacturers to produce the required quantities of granular product may need to be addressed given that these tropical legumes are very specific in their inoculum requirements. This will however be driven by demand for the product into the future. The alternative to clay granules could be to inoculate inert carriers (e.g. dead cereal grain seed) or live seed (e.g. silk sorghum) that can be placed at greater depth.

**Determination of inoculum survival in storage**
Many seed companies retailing tropical legume seed are supplying seed with rhizobium in the coating to producers for their grass/legume pasture sowings. There are questions regarding the storage of inoculum following application and up until sowing, given the seed is transported and stored in sheds, thereby subjecting the inoculum to fluctuating temperatures that are often higher than is recommended for good storage and survival. This seed is often stored by seed companies and/or producers for many months and not sown until the following season.

There is a need to conduct R&D to compare commercial seed coating inoculum survival over time following application to determine the percentage viable at sowing. This could be conducted in germination cabinets at intervals to determine whether the coating does in fact protect the inoculum.

**Provision of information to industry on inoculum requirements**
There is a perception in the pasture industry that inoculation is not necessary and that legumes will "pick up" a native bacteria and nodulate effectively. These native bacteria may form nodules but could limit the nitrogen fixing ability of the plant thus reducing its production potential.

There is a requirement to educate the seed industry, producers and their advisors that inoculation is critical and that there are improved methods available of introducing inoculum when sowing legumes.

**Determination of neptunia as a host of compatible rhizobia for desmanthus**
Our research suggests that CB3126 was not very effective at fixing N and gave no advantage in growth of desmanthus compared to applied N. Work on a potentially more effective strain could benefit desmanthus growth in our tropical pastures. There is limited research suggesting that the native legume neptunia (*Neptunia gracilis*) is a satisfactory host for rhizobia that can effectively nodulate desmanthus. There are some preliminary studies being conducted by James Cook University testing this theory. This will lead to a number of questions that will need refining and/or answering:

- If the native legume neptunia is growing in grass pastures do you need to inoculate desmanthus?

- How much neptunia needs to be growing in the paddock before inoculation is not required?
- Are there differences in the ability of the neptunia species or bio-types to nodulate effectively with desmanthus?
- Could there be a better strain of bacteria growing on neptunia than the currently available CB3126 strain recommended for both desmanthus and leucaena?
- The commercial strain CB3126 was collected from acid soil sites where as the neptunia strains are growing on alkaline soils where desmanthus and leucaena are recommended to be planted.

**Determination of the the capacity of the Warra soils to nodulate Caatinga effectively**
As stated previously in this report, there are very few soils that contain inoculum that will effectively nodulate Caatinga stylo. In the second pot trial and the irrigated field site there is evidence that the soil at the Warra trial site has a native bacteria that produces more nodules on Caatinga stylo than the current commercially available strain. This increased nodulation did not equate to increased dry matter production. It may be worthwhile investigating whether this nodulation data is repeatable and whether this native bacteria is superior to the commercial CB3481 strain at forming nodules on Caatinga stylo, and could lead to increased dry matter production.

**Effectiveness of alternative host legumes or other carriers of inoculum**
A potential alternative avenue to address the issue of the need for placement of inoculant at depth may be the use of a larger-seeded legume (therefore with greater seedling vigour and capacity to germinate from depth), wheat seed or silk sorghum that has the capacity to host the appropriate rhizobia for the smaller seeded legumes such as Caatinga stylo and desmanthus that are adapted to the region. If a suitable host species of legume or other carrier could be identified, it could be sown to depth at a low density, while the target small-seeded legume could be sown closer to the surface. The small seeded plant could be inoculated as they develop by the bacteria from the host plants as they develop.

The results from the field trials, indicate increased dry weights and nitrogen contents from the application of commercial inoculum via granules or water injection (this is more evident in the Caatinga stylo treatments). These methods of introducing rhizobia onto legumes are used in cropping systems and could be adapted and implemented when sowing legumes in grazing systems. The increase in legume dry matter production would improve animal production through increased protein in the diet and increased grass production and quality.

The Department of Agriculture and Fisheries pastures team in Toowoomba is already using water injection through a planter when sowing legumes in order to improve nodulation. These results will be delivered through workshops on introducing legumes within the "Improving productivity of rundown sown grass pastures" project (B.NBP.0639).

# 7 Key messages

The recommendations from this project are that inoculant survival is increased by delivering the rhizobia at depth in the soil where temperatures are lower and moisture higher, while still sowing the small seed close to the surface. These findings reinforce the need to correctly inoculate legume seed at sowing. There would be an improvement in survival and effectiveness if the rhizobia inoculum were placed at a greater depth in the soil than the seed.

One methodology currently used in cropping systems is to water inject the rhizobia at depth in the soil. This technology could be adapted to pastoral systems where machinery is used when sowing legume seed.

Over a range of soils types collected across southern and central Queensland, inoculation improved legume growth (particularly Caatinga stylo) and nitrogen content. This highlights the current departmental recommendations that these legumes should always be inoculated at sowing despite the contrary recommendations by some seed companies.

Productivity decline is a major issue with sown pastures. Research suggests that legume establishment offers the most cost effective long-term remediation strategy for addressing the issue. In order for legumes to make effective contributions to grazing systems they must be well-nodulated with effective rhizobia. Any industry and research progress towards improvements to bacterial survival and nodule formation will thereby improve N fixation and maximise the benefit from the legume component. This will have numerous flow-on effects including improved grass production, improved animal production, increased ground cover, reduced runoff, all of which provide strong justification for continued research efforts on legume rhizobial effectiveness.

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