**Title:**

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**Introduction**

For my half-semester placement, I worked under Dr Elena Colombi as part of her “*Functional integration of nitrogen-fixation in Australian indigenous rhizobia to improve soil fertility*” project. This project ultimately aims to identify the influence of genetic background on a soil bacteria’s ability to acquire and utilise rhizobia symbiosis genes. GENERAL BACKGROUND INFO on ICEsand IMEs

As Dr Colombi’s project is still in its initial stages, this placement involved on conducting preliminary tests and experiments with the aim to optimize the study’s experimental design. This was attempted from two different angles. First, to determine the symbiosis and nitrogen-fixing ability of *Mesorhizobium australicum* BR1-1-5xICE in different *Lotus* spp prior to the planned evolutionary experiment. Second, to determine a reliable method of confirming the presence of IME’s conferring symbiosis and nitrogen-fixation in *Pseudomonas* spp. Once confirmed, these bacteria would serve as donors to provide IME to non-symbiotic soil rhizobia via horizontal gene . As the IME is believed to carry copper (Cu) resistance gene, this study aimed to establish whether IME presence can be selected for by a Cu minimum inhibitory concentration (MIC) test.

CLOSING STATEMENT WITH EXPLICITLY STATED HYPOTHESES

1. *Pseudomonas* spp possessing IME will have greater Cu MIC than those that do not, thus confirming the presence of the IME
2. Symbiosis rates will differ between different *Lotus* spp., as different species and genotypes are very selective of their rhizobial symbionts
3. Any plants that do display symbiosis will have greater mass than those that do not, due to nitrogen fixation
4. Plant weights will vary between the different *Lotus* spp.

**Methods**

***L. australis* and *L. corniculatus s*eed inoculation**

Four seed samples were selected for inoculation: Two samples of *L. australis*, *L. australis* SA17133 (L2) and *L. australis* ID:#3 (L4), and two samples of *L. corniculatus, L. corniculatus* FRANCO (L3) and *L. corniculatus* HAM 216 (L5). Seed samples were supplied by Australian Pastures Genebank.

*L. australis* and *L. corniculatus* seeds were scarified by immersion in 98% pure sulfuric acid for 12 minutes, followed by five washes of sterile dH2O. Seeds were then immersed in 3.2% bleach for 20 minutes, followed by another five washes of sterile dH2O. Sterilised seeds were placed on fresh 0.8%(w/v) water agar and incubated in the dark at room temperature for 2 days. Successfully germinated seeds of L2 (n=9), L3 (n=4) and L4 (n=10) were transferred to test tubes with fresh 23mL Jansen agar slopes. Tubes were capped with foam plugs that allowed for gas exchange and incubated in the dark at room temperature for 2 days prior to inoculation.

*M. australicum* BR1-1-5xICE colonies from glucose Rhizobium Defined Medium (G/RDM) (no vitamin) plates were suspended in sterile dH2O and diluted to OD600 = 0.12 (2sf). 200µL aliquots of inoculum were pipetted directly onto seeds of L2 (n=6), L3 (n=3) and L4 (n=7). A subset of each seed Group was omitted from inoculation to act as negative controls (L2: n=3, L3: n=1, L4: n=3). Inoculated slopes were left to dry in lateral-flow cabinet for 40 minutes. Slopes (inoculated and negative controls) were wrapped in foil and transferred to a PC2 CO2 chamber incubate for 2 months.

**Symbiosis assessment**

*L. australis* ID#3 (L4), *L. uliginosus* GLOC005 (L7) and *L. rectus* Tas2206(L8) seeds were inoculated with *M. australicum* BR1-1-5xICE using the same protocol as described above by Dr Colombi prior to the initiation of my placement. All seeds were supplied by Australian Pastures Genebank. The *L. rectus* Tas2206group was incubated for 43 days prior to nodulation assessment. The sample set was composed three negative controls (L8-1, L8-2 and L8-3) and seven inoculated germinates (L8+1 – L8+7). The *L. australis* ID#3 and *L. uliginosus* GLOC005groups were incubated for 62 days prior to nodulation assessment. The *L. australis* ID#3 group consisted of four negative controls (L4-1 – L4-4) and six inoculated germinates (L4+1 – L4+6). The *L. uliginosus* GLOC005 group consisted of three negative controls (L7-1 – L7-3) and seven inoculated germinates (L7+1 – L7+7).

*Lotus* spp. shoots were harvested by careful removal them from the Jensen agar slope. Arieal cuttings of *L. australis* ID#3 and *L. uliginosus* GLOC005were dried at 75ºC for 2 days, and 4 days for *L. rectus* Tas2206, before weights were recorded. During the weighing process, subject L4+1 was misplaced before its weight could be recorded. As such, this subject was removed from later statistical analysis tests and calculations.

The establishment of symbiosis was assessed by counting and delimitating between well-developed nodules and smaller bumps presenting on the roots.

**Colony assessment**

Nodules were cut from the roots of all successfully nodulated subjects. This consisted of L4+1, +6 and +7 for *L. australis* ID#3 and L8+2, +3 and +5 for *L. rectus* Tas2206. In the case of L4+2 and the *L. uliginosus* GLOC005 subjects L7+2,+3 and +4, root cuttings displaying bumps were also harvested for analysis. In the case of the *L. uliginosus* GLOC005 subjects, this was deemed especially appropriate as no subjects displayed fully developed nodules, but a few possessed a considerable array of bumps.

Nodules and bumps were scarified by immersion in 80%(v/v) ethanol for 1 minute, then 3% bleach for 30 seconds, followed five washes of sterile dH2O. They were then immersed in sterile dH2O and crushed with a sterile pestle. Loopfuls of the resulting suspension were streaked onto G/RDM (no vitamin) plates and incubated at 28ºC for 5 days for *L. rectus* Tas2206, and 4 days for *L. australis* ID#3 and *L. uliginosus* GLOC005.

G/RDM plates inoculated with *L. rectus* Tas2206 suspension were assessed after 6 days of refrigerator storage. *L. australis* ID#3 and *L. uliginosus* GLOC005 plates were assessed directly following incubation. In all instances, colony morphology was qualitatively assessed and compared across different the plant subjects.

**Statistical analysis**

Analysis of the relationship between symbiosis (nodule/bump formation) and dried weight was investigated using R Studio1 and Microsoft Excel2. The nodulation and bump counts for the subjects of each *Lotus* spp were graphically summarised in collated bar plots. The average number of nodules and bumps were each symbiotically-successful subject were also summarised in a bar plot to illustrate similarities and differences between species.

To investigate whether the symbiosis resulted in nitrogen fixation, the weights of different plant subjects were grouped by symbiotic state (no nodules/bumps, nodules and/or bumps, nodule presenting, bumps only). The mean (±95%CI) weights were used to construct a clustered bar plot for visual comparison. The impacts of nodulation/bump state and plant species on mean weight were each separately assessed in separate One-Way ANOVA’s.

To assess the validity of analysis results, the ANOVA assumptions were also tested. Homogeneity of variance (assessing the null hypothesis that the variance is equal across) was assessed by a Levene’s Test. This was further tested by plotting residuals against fitted values and assessing the fitted line. The assumption of normal data was tested by assessing the linearity of a Normal Q-Q plot. The third assumption, the independence of observations, was addressed in the experimental design. Each seedling was grown on a separate Jensen agar slope, with each slope isolated in a separate sterile test tube. This experimental set-up ensured that the growth of one plant did not affect the growth of another.

**Cu MIC Assay**

*Trial I*

Four strains of *Pseudomonas* spp. were assessed to determine their Cu MIC: *P. protegens* Pf5, *P. stutzeri* 17587, *P. stutzeri* 17641 and *P. stutzeri* Q. Strains 17641 and Q acted as positive controls (possess an IME of interest) while strains Pf5 and 17587 acted as negative controls (lack the IME).

*P. stutzeri* strains were incubated at 28ºC on LB 1.5% agar media for 2 days, and *P. protegens* Pf5 for 1 day, prior to Cu MIC assay. Loopfuls of colony from each plate were resuspended in 10mM magnesium sulphate. Each cell suspension underwent a 1:10 series dilution to generate a set of solutions of decreasing concentration, with a range of 1:100 - 1:107. 20µL aliquots of each dilution were dispensed across a series of fresh Cu MGY 1.6% agar plates. Cu concentrations of 0mM, 0.8mM, 1mM, 1.3mM, 1.6mM and 2mM were used in for MIC assessment. The inoculated plated were incubated at 28ºC for 2 days prior to MIC assessment.

*Trial II*

Due to unexpected results from the first MIC and following a successful quality test of the Cu MGY 16% agar media, the MIC assay was repeated. The experiment was set up as described in *Trial I*, with an adjustment to the Cu concentrations in the media. This assay employed a range of 0mM, 0.3mM, 0.6mM and 1mM. Plates were incubated at 28ºC for 2 days, then moved to the 3ºC refrigerator for storage. Plates remained in the refrigerator for 2 days prior to assessment. Both qualitative and quantitative assessment of colony growth was recorded.

**Cu MGY media quality assessment**

Following unexpected results in Cu MIC *Trial I*, a quality control assay was conducted to determine if the odd results were a result of poor plate preparation

Control colonies were sampled from previously prepared cultures supplied by another member of the EMMA lab group (Ask Elena for her name!). For a negative control, 8 colonies of the Cu-sensitive phyllosphere culture "Phyllo B" grown on 0mg/L Cu *Pseudomonas* Selective Media (PSM) were randomly selected. For positive controls, 3 colonies from the "Phyllo-C" phyllosphere culture grown on 300mg/L Cu (equiv. to 4.72mM) PSM, 3 colonies from the "Phyllo-C" phyllosphere culture grown on 200mg/L Cu (equiv. to 3.15mM) PSM, and 2 colonies from the "Rhizo-C" rhizosphere culture grown on 200mg/L Cu PSM were randomly selected. The positive and negative control colonies were streaked on 0mM, 0.8mM and 1.0mM Cu MGY 1.6% agar plates (from *Trial I* plate preparation). Plates were incubated at 28ºC for 2 days prior to growth assessment.

***Lotus* spp growth conditions**

* All *Lotus* spp seedlings were incubated in a PC2 CO2 incubation chamber. CO2 level: general atmospheric conditions. Relative humidity: 70%. Day/Night cycle: Day: 16hrs, 22ºC, standard white light. Night: 8hrs, 14ºC, no light. No watering needed.

**Media preparation**

*Jensen media* (per 1.00L): CaHPO4 (1.00g), K2PO4 (0.20g) MgSO4⸳7H2O (0.20g), NaCl (0.10g), FeCl3 (0.10g), Agar (12.0g).

*0.8% water agar* (per 400mL): Agar (3.2g)

*G/RDM (no vitamin) media* (per 200mL): L-Histidine (20.0mg), G/RDM salts (2.5% MgSO4⸳4H2O, 0.2% CaCl2⸳2H2O, 0.7% FeEDTA, 2% NaCl) (2.00mL), 0.2% Bromothymol-Blue (2.00mL), 1.8% NH4Cl (1.15mL), Agar (3.20g), “Trace Elements 4 GRDM” (200µL)

*Cu (xmM) MGY media* (per 600mL): Mannitol (6.00g), L-glutamic acid (1.20g), KH2PO4 (0.30g), NaCl (0.10g), 1.0M KOH (adjusted to pH 7.0), Yeast Extract (0.15g), Agar (9.6g), 1.0M CuSO4 (100µL per 1mM concentration required)

**Results**

**Colony assessment**

All plates inoculated with the nodule suspension prepared from subjects L8+2, +3 and +5 displayed bacterial growth. Each isolate possessed a similar morphology of green ill-defined wet colonies, with no secondary isolates or contaminants observed. The fact colonies appeared identical across nodules from all three assessed plants is strong evidence that the recovered isolate is the same bacteria used in the initial seedling inoculation, *M. australicum* BR1-1-5xICE. This displays that the bacteria was able to successfully utilise the symbiosis genes of the ICE to colonise the roots of *L. rectus* Tas2206.

In contrast, the plates inoculated with the suspension from *L. australis* ID#3 and *L. uliginous* GLOC005 root cuttings displayed no growth. This indicates that although symbiosis initially began (as evidenced by the nodules/bumps), the plant eventually rejected the *M. australicum* symbiont at a later point during nodulation3. Without the nutrients from the host plant, the bacteria died in the nodule.

**Nodulation assessment**

*L. rectus* Tas2206

Of the seven *L. rectus* Tas2206 seedlings inoculated with *M. australicum* BR1-1-5xICE, five displayed any signs of rhizobia symbiosis. All five presented with small orange bumps, which may be evidence of the beginnings, or an attempt, at nodulation. Of these five, only three plants presented with developed nodules (L8+2: 1, L8+3: 4, L8+5: 3) (**Figure N**). When considering both nodules and bumps, *L. rectus* Tas2206 had an 88.9% success rate of establishing a symbiosis.

*L*. *australis* ID#3

Of the seven *L*. *australis* ID#3 seedlings inoculated, four displayed signs of rhizobia symbiosis. L4+2 and L4+7 each presented with 1-2 small green bumps, which may be evidence of the an attempt at nodulation. Three subjects presented with developed nodules (L4+2: 1, L4+6: 2, L4+7:1). (**Figure N**). Unlike *L. rectus*, only one of the *L*. *australis* nodule-presenting plants also displayed a bump (L4+7). While 55.6% if the inoculated subjects displayed signs of symbiosis, it would be inaccurate to report this as a “success rate” as no *M. australicum* was recovered.

*L. uliginosus* GLOC005

None of the seven inoculated *L. uliginosus* GLOC005 subjects displayed nodules. However, four subjects displayed a series of small orange bumps (L7+2: 10, L7+3: 10, L7+4: 26, L7+5: 9) (**Figure N**). The frequency of these bumps suggests multiple *M. australicum* cells attempted to form a symbiosis, however none were particularly successful.

**Weight assay**

When considering weight as a function of symbiosis state (no nodules, nodules and/or bumps, nodule presenting, bumps only) the data successfully met all ANOVA assumptions. Homogeneity of variance was mostly confirmed by the relatively flat line in the Residuals vs Fitted Values plot **(Supp.Figure N).** The sudden shift at the midpoint displays that there are two slightly distinct trends in the variance. However, the implications of this could not be interpreted, and so a Levene’s Test was conducted to further test variance homogeneity. This test did not reject the null hypothesis (F3,8 = 0.250, p = 0.8591), thus supporting the decision that the variance is reasonably homogenous. Normality of data was also confirmed by reasonable degree of linearity in the Normal Q-Q plot **(Supp.Figure N).**

The One-Way ANOVA found no significant interaction between symbiosis state and weight of the cuttings (F3,8 = 0.145, p = 0.9300). This indicates that the symbiotic *M. australicum* BR1-1-5xICE did not provide its plants with nitrogenous compounds (or at least, not enough to significantly benefit its host’s biomass). Thus, it appears that the bacterium was incapable of utilising the N+ genes of the ICE.

[When considering weight as a function of plant species (*L. australis*, *L. rectus* and *L. uliginosus*) the data successfully met all ANOVA assumptions. Homogeneity of variance was assured by the flat line in the Residuals vs Fitted Values plot **(Supp.Figure N).** This was further supported by a Levene’s Test which did not reject the null hypothesis of homogenous variance (F2,9 = 0.138, p = 0.8732). Normality of data was also confirmed by the reasonably linearity in the Normal Q-Q plot **(Supp.Figure N).**

The One-Way ANOVA found a significant interaction between plant species and weight of the cuttings (F2,9 = 8.77 p = 0.0077). This indicates that each *Lotus* spp. Assessed is unique in its growth rate. Such differences in mass are likely to reflect other differences between the species, such as the signalling peptides and flavonoids involved in forming rhizobial symbiosis4. As such, it is important to recognise that as these species differ in their biochemistry, the differences in nodulation patterns described in *Nodulation assessment* are almost to be expected. (Is this valid, or am I just talking shit rn)]

**Cu MIC Assay**

*Trial I* of the Cu MIC assay produced unexpected results. While they displayed good growth on the 0mM plate, no growth was observed on plates with Cu ≥ 0.8mM. However, the subsequent quality assessment of the media went as expected. The Cu-tolerant colonies sampled from Phyllo-C (grown on Cu 300mg/L), Phyllo-C (grown on Cu 200mg/L), and Rhizo-C (grown on Cu 200mg/L) all displayed good growth on 0mM, 0.8mM and 1.0mM plates. Moreover, the Cu-sensitive "Phyllo B" colonies grew well on the 0mM plate, but displayed minimal to no growth on the 0.8mM and 1.0mM plates, as expected. **(See Supplementary**). These results display that absence of growth in *Trial I* was not the result of faulty MGY preparation, but instead an indication that the lowest Cu concentration of 0.8mM was above the Cu tolerance level of the isolates.

**Table 1**. Cu MIC *Trial II* results. MIC measures are based on the 1:107 diluted inoculation patch of each bacteria’s series dilution. The *Pseudomonas* species of the “strains” listed refer to *P. protegens* for Pf5, and *P. stutzeri* for 17578, 17641 and Q. Strains 17641 and Q are carries of the IME of interest, while Pf5 and 17578 are negative controls.



*Trial II* of the Cu MIC also produced unexpected, albeit interpretable, results. As displayed in **Table 1**, the four strains varied in their tolerance to Cu. When comparing the most diluted inoculation patch of the dilution series, 1:107, *P. protegens* Pf5 displayed the greatest resistance (MIC: 0.6mM), followed by *P. stutzeri* 17587 and *P. stutzeri* Q (MIC: 0.3mM), then *P. stutzeri* 17641 (MIC 0.0mM). These results support the hypothesis that the absence of growth in *Trial I* was largely due to testing Cu concentrations above the tolerance level of the isolates. However, it is important to note that while no dilution patches grew on any plates with Cu ≥ 0.8mM, some of the more densely concentrated inoculation patches for *P. protegens* Pf5, *P. stutzeri* 17587 and *P. stutzeri* Q displayed MIC’s of >1.0mM. **(See Supplementary**). *P. stutzeri* 17641 also displayed MIC’s of 1.0mM for some of its more densely concentrated patches . **(See Supplementary**). Therefore, an error in plate preparation for *Trial I* may be partially to blame for these inconsistencies.

It should be noted that the Trail II MIC for *P. stutzeri* 17641 was complicated by contamination. While the initial inoculation patches appeared as expected (small translucent colonies), the 1:105 patch possessed large fluorescent colonies. These colonies were immediately deemed as odd, as *P. stutzeri* is typically distinct from many other *Pseudomonas* spp. specially due to it’s lack of fluorescent pigments5. However, these colonies appeared near identical to those of *P. protegens* Pf5 and so the abnormality was attributed to an error in preparation of the serial dilution that resulted in contamination. However, it appears that this preparation error may have also resulted in solutions for 1:106 and 1:107 missing their inoculants. This would explain the surprisingly low MIC of 0mM for the *P. stutzeri* 17641 1:107 patch. However, even without this error it is unlikely that the Cu MIC results would have indicated anything much higher. All higher inoculation patches had displayed little growth by Cu 0.6mM, and absolutely no growth on Cu 1.0mM. As such, it is reasonable to assume that *P. stutzeri* 17641’s true Cu MIC would be something similar to the other IME carrier, *P. stutzeri* Q, with a 1:107 Cu MIC od 0.3mM.

**Discussion**

**Conclusions**

**Data Management Plan**

**References**

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