GLOBE International Virtual Science Symposium 2017

**There’s Something In The Air: Developing A Diazotroph-Based Biofertilizer**

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**Abstract**

**Key words: nitrogen fixing bacteria, biofertilizer, nitrogen cycle**

Current use of synthetic fertilizers significantly disrupts the nitrogen cycle. For instance, nutrient runoff and leaching harm fragile ecosystems and contribute to climate change.

As a more sustainable, environmentally-friendly alternative to synthetic fertilisers, we are

investigating the use of diazotrophs in biofertilizers. Our biofertilizers consist of cultured diazotrophs from six different Rotorua locations. Mung beans, *Pinus radiata* and radishes were inoculated with these diazotrophs. We also tested a variety of commercial fertilisers. The test plants were allowed to grow from seed for approximately two months, including initial germination and maturation. Plant heights were measured every 10-14 days and at the end of the experiment, dry weights of the full plant were taken. We also conduced genetic analysis of the diazotrophs from the soil samples and were able identify some of them.

After one-way analysis of variance (ANOVA) of our data, it was found that there was no

statistically significant difference between treatments. This may have been due to experiment length, climactic conditions or a small sample size. However, there is a growing body of scientific evidence that suggests that biofertilizers consisting of nitrogen fixing bacteria positively impact plant growth, and can be a substitute or supplement to conventional nitrogen fertilizers.

**Research questions and hypothesis**

The aim of our project is to investigate the efficiency of four different biofertilizer mixes consisting of nitrogen fixing bacteria isolated from six different soil samples collected in the Rotorua region. These mixes will be compared with a range of existing commercial fertilisers to determine which fertilizer maximises plant height and biomass in a variety of plant species (mung beans, radish and *P. radiata*).

This is an important question to answer because

Our hypothesis is, “If we apply our own biofertilizers mixes derived from soils around Rotorua, *P. radiata*, mung beans and radishes will experience increased plant height growth and biomass in comparison to synthetic commercial fertilisers and the control.” There are many studies that show biofertilizers increase plant growth and biomass, so we have confidence in this statement.

Based on research into soil types, we predict that the biofertilizer containing nitrogen fixing bacteria isolated from Kuirau Park will be most effective. This is due to the unique bacteria that live in the warm, acidic, geothermal conditions at that location. Biostart-N will be the most effective out of the commercial treatments as this was the most highly priced product, and contained a single pure strain of Azotobacter chroococcum. This bacteria has been shown to significantly increase crop yields (Wani. S , et al 2013).

**Literature Review*:***

Nitrogen cycle:

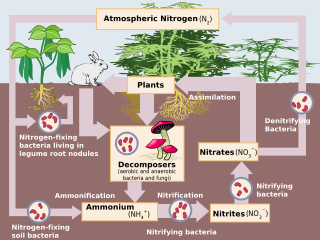
Nitrogen is an essential component of nucleic acids and proteins which are the ‘building blocks’ which make up all living things. There is only a limited amount of nitrogen on the earth so the continuation of life depends on the nitrogen cycle. This cycle describes the conversion of nitrogen between different chemical forms and its movement between reservoirs. Bacteria and other microorganisms play a key role in the nitrogen cycle as they facilitate the movement of nitrogen by carrying out some of its chemical conversions (Lamb, J., Fernandez, F et al).

Figure 1: Diagram of nitrogen cycle

The atmosphere, consisting of about 78% nitrogen, is a major nitrogen reservoir. However, most organisms cannot directly assimilate nitrogen in this form, as a high amount of energy is required to break the strong triple bond in N2 (bond energy of 941 kJ/mol). Certain prokaryotes are able to convert atmospheric nitrogen to organic forms that are then accessible to the ecosystem (International Plant Nutrition Institute)*.* This process is called *nitrogen fixation*. It is estimated that biological nitrogen fixation is responsible for fixing about 175 million metric tons of nitrogen globally per year (Bezdicek & Kennedy).

Ecosystem decomposers convert the organic nitrogen in biological waste and remains to ammonium (NH4+) by the process of *ammonification*. Most of the ammonium produced by aerobic decomposition is quickly assimilated again by organisms, but in anoxic conditions ammonium remains stable within the soil. As ammonia is a highly volatile compound, it can be lost from soils by vaporisation, particularly in highly alkaline, warm and/or moist conditions. Loss of nitrogen from soil in this way is a major issue in agriculture and can lead to economic losses as well as potential adverse health effects (Fowler, D., Coyle, M. et al). Globally however, ammonia volatilisation represents only 15% of nitrogen released to the atmosphere. The rest is attributed mainly to the denitrification process.

*Nitrification* is a two step process carried out once again by soil microorganisms. In the first step, ammonium is oxidised to nitrite (NO2-) which is then converted to nitrate (NO3-) in the second step. Plants then assimilate this nitrate. From an agricultural perspective, nitrification can pose an issue as the highly water soluble nitrate it produces easily leaches out of soils during high rainfall. Chemicals are often added to fertilisers in order to inhibit nitrification, with varying degrees of success.

When soil oxygen levels are low, some soil microorganisms use nitrate for respiration. As a product of this *denitrification* process, nitrogen is returned to the atmosphere in one of a number of gaseous forms, thus completing the nitrogen cycle. One of these gases, nitrous oxide (N2O), has been linked to ozone depletion and is a potent greenhouse gas.

**Biological nitrogen fixation**

There are a variety of prokaryotes including archaea and cyanobacteria that are capable of fixing nitrogen. These organisms are called diazotrophs. A table of bacterial diazotrophs is given below.

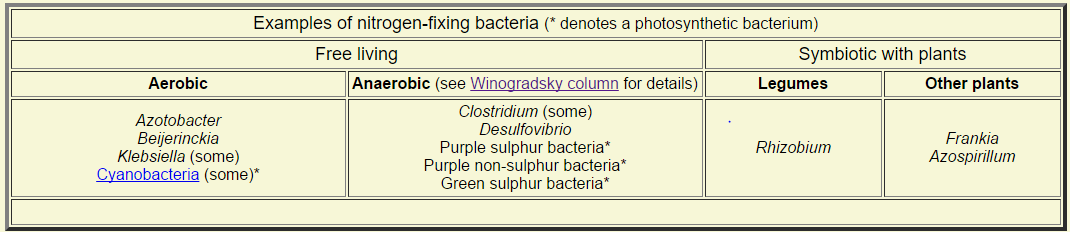
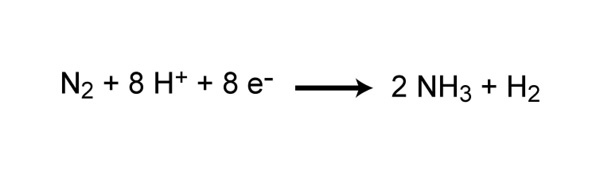


Figure 2: Table of nitrogen fixing bacteria

Nitrogen fixation is an energy-demanding process by which N2 is converted into ammonia. The overall reaction for this is shown below.



The enzyme complex nitrogenase catalyses this reaction. It consists of two separate proteins, dinitrogenase and dinitrogenase reductase, the latter of which is encoded for by the *nifH* gene. Nitrogenase activity is strictly controlled in order to optimise the metabolism of the bacteria. For instance, the enzyme is inactivated by oxygen and excess nitrogenous compounds. This regulation of nitrogenase activity allows for the balance of nitrates within the soil.

This study will be focusing on the symbiosis between nitrogen fixing bacteria and plants. A symbiosis is an interaction between organisms in which both individuals benefit from this mutualistic relationship. Each organism contributes to the survival benefit of the other and in turn receives its own survival benefit. In the case of plant-bacteria symbiosis, the plant gets a built-in supply of fixed nitrogen and the bacteria get a safe, well nourished habitat in the root nodules (Shamseldin, 2013). In some instances, stem nodulation also occurs.

The root nodulation process involves the exchange of chemical signals between the plant and bacterium. Bacterium then excrete rod factors and infect the root hair. Subsequent, growth and development leads to the formation of mature rood nodules. However, root nodulation is not a guarantee of a successful symbiosis. Depending on *nod* genes present, the bacteria may be of an ineffective strain and be unable to fix nitrogen. This is indicated by small, pale coloured nodules. An effective strain on the other hand will be able to fix nitrogen and produce large, reddish coloured nodules.

Human impacts on the nitrogen cycle

The Haber-Bosch process is the main industrial procedure used to produce ammonia today. Air is pumped into a large vessel and oxygen is removed by combustion. Carbon dioxide is also removed, leaving nitrogen and hydrogen. An electric current causes these two elements to react and form ammonia. High pressure (200atm) and temperatures (400°C) are required. The ammonia may then undergo further processing into other substances. The full equation is shown below.

N2 + 3 H2 → 2 NH3     (Δ*H°* = −91.8 kJ) => (Δ*H°* = −45.8 kJ·[mol](https://en.wikipedia.org/wiki/Mole_(unit))−1)

The invention of the Haber-Bosch process for producing ammonia revolutionised the world.

This form of artificial nitrogen fixation allowed for the mass manufacture of nitrogen-based

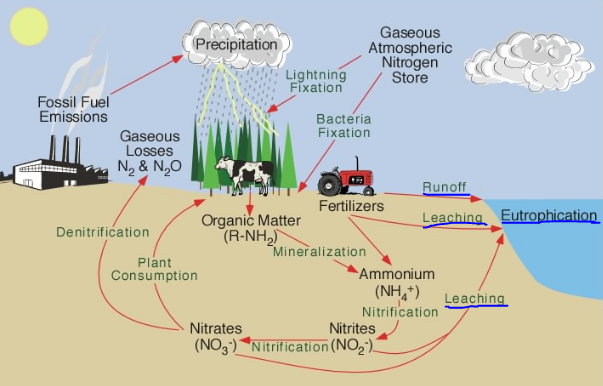
fertilisers which in turn led to huge increases in global crop yields and supported a rise in global population. For some, nitrogen fertilizers help make the difference between malnutrition and an adequate diet. In fact, half the protein in our bodies is thought to contain nitrogen fixed through the Haber-Bosch process. However, current fertilizer use is inefficient and unsustainable with the world using 177 million tonnes of fertilizer annually. This contributes to negative environmental effects such as eutrophication.

Figure 3: Factors leading to eutrophication

Eutrophication is a natural process in which aging bodies of water become nutrient rich, leading to the excessive growth of plants and algae. This is due to the increased availability of one or more growth limiting factors required for photosynthesis. This occurs naturally over extended periods of time as bodies of water are filled in with sediments and due to nutrient build up from runoff (Charles, 2013). Cultural or anthropogenic eutrophication occurs due to human activities, which increases the rate and extent of eutrophication. This occurs through non-point loadings of limiting nutrients, specifically nitrogen and phosphorus (Carpenter et al), and point source discharges into aquatic ecosystems (refer to Figure 3). Intensive agricultural practices are usually the source.

These excess nutrients stimulate algal blooms of organisms such as cyanobacteria, phytoplankton, duckweed and other aquatic plants (usually unfavourable organisms). The proliferation of these plants causes biomass imbalance in trophic levels. These dense algal blooms limit light penetration which causes plants in the littoral zone to experience reduced growth and eventually die off. This also decreases the chances of survival for predators that require light to pursue and target prey. Water clarity and quality is also reduced due to the proliferation of phytoplankton which can impact recreational activities (Wilson, A. E. et al)*.* Furthermore, during the day dissolved inorganic carbon is depleted due to high photosynthetic rates of aquatic plants (see algal bloom), increasing pH levels. This can increase toxicity of other substances, and can kill marine life and poison land animals.

Once algae die, decomposition of the organism consumes oxygen, reducing the dissolved oxygen concentration in the water (Richards, 2009)*.* This can lead to hypoxia (inadequate oxygen levels reaching tissue) in marine animals which results in organisms having difficulty maintaining homeostasis. This causes decreased fish yields in fisheries (due to oxygen depletion), and reduces the amount of desirable fish species. If conditions become anaerobic this can promote bacterial growth of *Clostridium botulinum*, which produces toxins deadly to mammals and birds. Consequently, dead zones are created, causing a loss of biodiversity.

On a local level (Bay of Plenty area and surrounding), eutrophication has affected water quality in several Rotorua lakes. Water quality has declined with the development of land for agriculture and with the increasing fertilizer applications. Lake Okaro, for example, was categorised as eutrophic (Regional Land and Water Plan, 2004).

Point and non-point loading of nutrients are sources of nutrient runoff which contribute to anthropogenic eutrophication. In point sources, nutrient waste travels directly to a water source (e.g. lakes and streams). Examples of point sources include runoff from animal feeding lots and sewage overflow. Nonpoint loading comes from many different sources, and is more difficult to regulate. Examples include runoff from agriculture, irrigation, and pastures. Through our study into the application of nitrogen fixing bacteria, we propose a method to limit and control non-point loading such as nitrogen leaching and runoff.

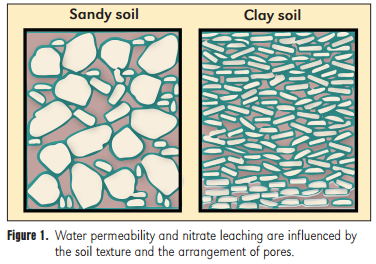
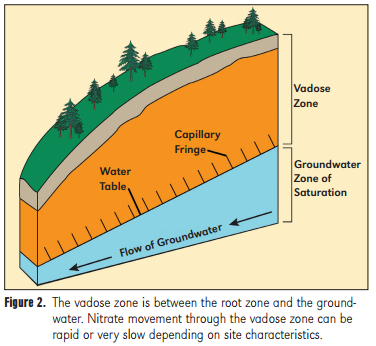
**Nitrogen leaching is the loss of water soluble nitrate as it moves through soil below the root zone. When the soil’s water holding capacity is exceeded, this facilitates the movement of soil nutrients through and from soil. This can lead to nitrates entering ground or surface water, contributing to eutrophication (Belnap). Nitrogen leaching involves the following three main factors: 1) presence of nitrogen in soil, 2) the permeability of soil and 3) water movement through soil. Soil and bedrock conditions also play a large role in nitrogen leaching. Nitrate is susceptible to leaching and transformation, therefore the amount of nitrate in the soil should be limited to the requirements of the plant.

Figure 4: Soil factors such as composition and texture can influence levels of nutrient leaching

Soils of fine texture (high clay) are less susceptible to nitrate movement, however are more prone to denitrification losses. The higher the nitrogen leaching vulnerability of a soil, the more likely nutrients will enter bodies of water through runoff. The vadose zone is unsaturated, it lies beyond the root zone and above the ground water zone. Correlation is shown between agricultural practices that occur on the surface of the soil, and the N transformations in the vadose and ground water zones *(Belnap, J).*

Agricultural practices involve applying nutrients onto fields in order to facilitate plant growth and maximise production (this aspect of agricultural practices is the focus of our study). However, through the frequent application of nutrient mixes, urea and nitrogen fertilizers this leads to a surplus of nutrient present in the soil (especially within the root zone), which will leach out of the soil and runoff into bodies of water. Agricultural practices play a large role in the contribution of non-point loadings of nutrients.

Figure 5 The vadose zone is between the root zone and the groundwater. Nitrate movement through the vadose zone depends on site characteristics.

According to Rockström *et al*. (2009)and Steffen *et al*. (2015), the world has already overstepped its planetary boundaries of the nitrogen cycling process (i.e. an excess of 35 million tonnes of nitrogen is being removed from the atmosphere each year). Thus, this is an urgent issue that needs to be addressed.

Test plants

*Pinus radiata* is found in the Pinaceae familyand is a fast growing softwood. It is a coniferous evergreen tree and can reach up to in 60 m height in optimum conditions and in the wild grows approximately 15 to 30m in height. New Zealand’s plantation forests are 89% of this species. *P. radiata* grow well in sandy, loamy, well drained soil. Requiring moist or dry soil, and can grow in nutritionally poor soil. High light intensity is preferred. We decided to use *P. radiata* in our investigation as it will give our project a direct link with NZ forestry and possible related applications. The other test species were chosen mainly due to convenience and availability.

Radishes are a root vegetable found in the Brassicaceae family. The seed germinates in 3 – 4 days in moist conditions with a soil temperature of 18 – 29 °C. They grow best in a pH of 5.8 to 6.8 (weakly acidic/slightly below neutral) and favour areas of high light intensity, with loose and well-drained soil. The radish roots will bifurcate around rocks within their growth path. Radishes require frequent watering, even watering of the soil prevents radishes from cracking. Radishes are ready to harvest when their roots are approximately 2.5cm in diameter, this can take 22 - 70 days for maturity to be reached, varying between radish types.

Mung beans are found in the legume family and are a warm season crop, and takes a few weeks to reach maturity. They grow well in fertile sandy, loam soils and require adequate drainage. The optimum pH for growth is between 6.2 and 7.2 (weakly acidic/slightly above neutral). When planting mung beans, proper nitrogen fixing bacteria must be provided, the inoculant must be distributed uniformly. The bacteria used must be specific for mung beans or a closely related species (Bradley, S., Smith. F. et al).

**Research methods**

Planting Radish, *P. radiata* and Mung Bean Seeds

*Equipment:*

* V150 Container (24 cells)
* *P. radiata* Seeds
* Mung Bean Seeds
* Radish Seeds
* 50/50 Peat and Perlite Mix
* Water

*Method:*

1)      Fill the V150 container 50/50 peat and perlite mix, distributing the mix evenly throughout the 24 cells. The mix does not contain fertilizer or trace elements. This ensures that only the treatments applied after germination affect the growth of the *P. radiata*, mung beans and radishes.

2)      Water the soil mix well, then sow seeds (2- 3 seeds in each cell). Bury the seeds at no more than a 1.5cm depth in the centre of the cell.

3)      Repeat this process for all the mung bean, radish and *P. radiata* seeds.

Collecting Soil Samples

*Equipment:*

* Collection tubes (x6)
* Spade
* Labels

*Method:*

1)   Collect the top soil (1-10cm below ground) in the area selected. Do not collect soil out of a fertilised garden. The top soil is collected, as deeper down in the soil there are less aerobes (due to decrease in oxygen levels). Label each soil sample.

2)      Repeat this process when collecting soil samples from different areas around Rotorua. Make sure to collect all the soil samples within a 24 hour time frame.

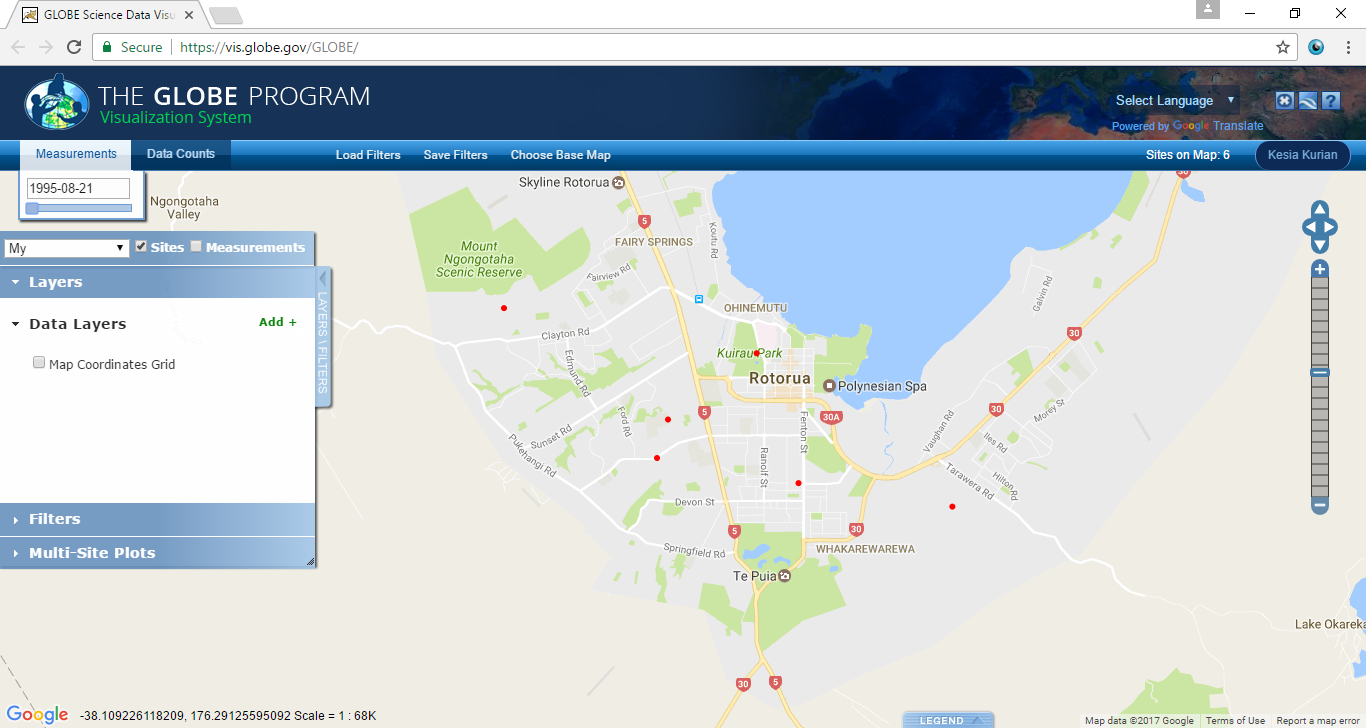
3)      After the soil samples have been collected remove all leaves, twigs etc. Then refrigerate the soil to keep the microorganisms alive.

Note: All equipment was washed between each soil sample collection and sifting in order to avoid cross-contamination.

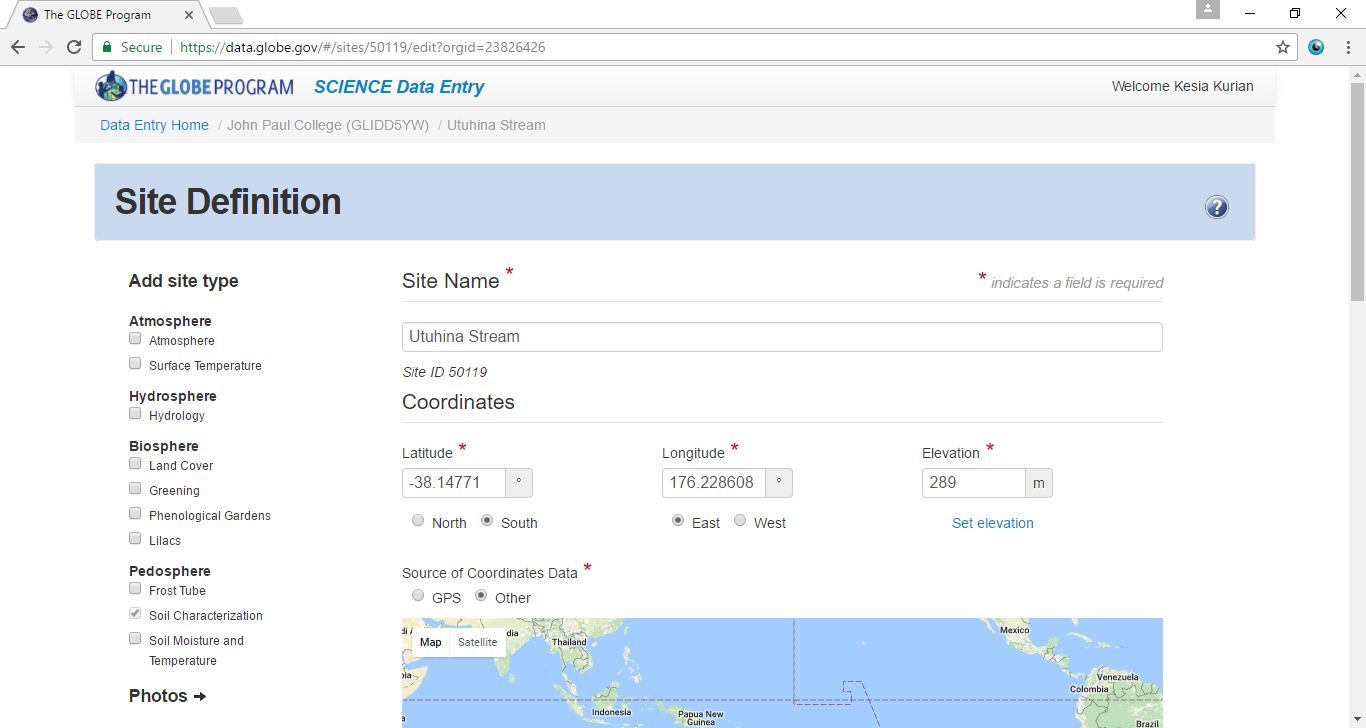
Note: In this experiment soil samples were collected from the following areas:

1. Ngongotaha/Western Heights
2. Red Woods
3. Glenholme
4. Utuhina Stream
5. John Paul College grounds
6. Kuirau Park

*Visualisation of sample sites (red dots) on GLOBE site*



*Data entry on GLOBE site*



**Making the NLMM (nitrogen limited minimal medium)**

Nitrogen limited minimal media (NLMM) was used to grow bacteria in and culture on agar based plates. This media does not contain a source of nitrogen, and all living organisms need nitrogen in order to survive. Therefore, only the bacteria that are able to fix atmospheric nitrogen are able to survive on the media. Other bacteria will die off. This isolates only the nitrogen fixing bacteria (and eliminates all other bacteria present), which allowed us to culture nitrogen fixing bacteria present from the different soils from around the Rotorua area to create our own bacterial ‘mixes’ / biofertilizers.

Yeast Extract (Final 50 mg/L concentration)

5 g in 250 ml milliQ water

Autoclave

KH2PO4                                            0.4 g

K2HPO4                                            0.1 g

MgSO4.7H2O                              0.2 g

NaCl                                                  0.1 g

FeCl3                                                  10 mg (mix with a small amount of water separately)

Na2MoO4.2H2O                             2 mg

Yeast extract                  2.5 ml

Add 950 ml of milliQ water and autoclave

Carbon source

Glucose                             5 g/L

Once cool add:

Filtered glucose             50 ml

Adjust medium to final pH of 7.2 + 0.1

Culturing bacteria from Soil Samples in NLMM

*Note: see ‘Appendix B – Justification of Decisions Made’ to read about our decision to use this specific growth media.*

*Equipment:*

* 250 mL conical flask
* Soil Samples
* Incubator
* Cellotape
* Pipette
* 100mL measuring cylinder
* Funnel
* Distilled water

Inoculating Flasks

1. Using a measuring cylinder, measure 100mL of media and pour into the conical flask.
2. Put 1 g of each soil sample into the corresponding flask and put on the lid.
3. Repeat this process for each different soil sample.

Incubating Flasks

1. Secure conical flasks to incubator tray using sellotape.
2. Turn on incubator and set to 30°C, increase the shaking speed incrementally until the medium shaking speed is reached.
3. Put tray with secured conical flasks into incubator and leave for two weeks.
4. Check the cultures every one to two days to observe growth. If cloudy, this shows bacterial growth.
5. Keep the incubator and its contents out of direct sunlight / in a shaded area.

Notes:

* Only open the tubes and bottles of media for as little time as possible. As soon as the bottles of media are open (to the atmosphere) it is not sterile.
* The lids on top of the conical flasks fit loosely to allow for oxygen to enter the flasks.
* 100mL of media was poured into the 250mL conical flasks to allow space for oxygen to enter the flask.
* The temperature of the incubator was set to 30°C as a higher temperature would prevent the growth of bacteria (denature proteins making up bacteria).
* Ridges in the bottom of the flasks allow for aeration to occur. This promotes bacterial growth.

Inoculating Treatments onto Test Plants

*Equipment:*

* Pipette
* Water
* Labels

*Method:*

1. Once the plants have germinated remove any excess plants so that only one plant is growing in each cell.
2. After two weeks of growth in NLMM the bacterial mixes are ready to inoculate, apply (pipette) the treatments (2.0ml each trial) to the radish, mung bean and *P. radiata* plants (4 trials for each treatment). Follow instructions specified for each commercial treatment.
3. Randomise the treatments throughout the cells for the radish, mung beans and *P. radiata*. Label each plant with the type of treatment applied and the trial number.
4. Measure the stem height of the plants each week and water every 2 to 3 days.

Culturing Bacteria

* Serial Dilution:

*Equipment:*

* 80% Ethanol
* Pipette Tips
* Pipette
* Nitrogen fixing bacteria (from soil samples)
* Agar
* Petri dishes
* Incubator

*Method:*

1. Clean laminar flow cabinet with 80% ethanol before use. This is a sterile environment, so change the pipette tip each time the pipette is taken out of the laminar flow cabinet.
2. Conduct serial dilution of each soil bacteria culture up to 10-8
3. Plate 10-4, 10-6 and 10-8 dilutions of each soil bacteria culture on agarbased NLMMPlates.
4. Store plates in an incubator set to 30°C

* Re-streaking Plates

*Equipment*

* 80% ethanol
* Agar
* Petri dishes
* Inoculating loop
* Incubator
* Nitrogen fixing bacteria (sourced from soil samples)

*Method:*

1. Clean laminar flow cabinet with 80% ethanol before use.
2. Pour agar into petri dishes and allow them to set.
3. Select an isolated individual colony of bacteria from the initial cultures and use inoculating loop to restreak it. (image of streaking pattern shown below).
4. Repeat step 3 three times for each bacteria sample, using a new loop for each colony.
5. Put original cultures back into refrigerator and put restreaked plates into incubator set to 30°C.

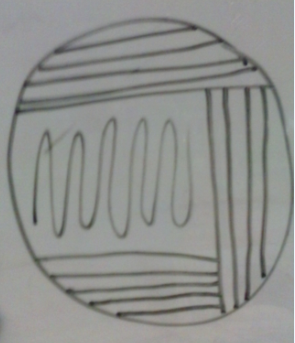
* Producing a Pure Strain from a Single Bacteria Colony (Isolation)

*Equipment*

* 80% ethanol
* Agar
* Petri dishes
* Inoculating loop
* Incubator
* Nitrogen fixing bacteria (sourced from soil samples)

*Method:*

1. Clean laminar flow cabinet with 80% ethanol before use
2. Pour agar into petri dishes and allow them to set.
3. After sufficient growth of bacteria, select a single colony of bacteria using an inoculating loop.
4. Re-streak the single colony of bacteria on an agar plate (pattern shown below).
5. Allow time for bacteria to grow.
6. This produces a pure strain from a single bacteria colony (isolation) that is suitable for use in a DNA extraction.



Note: keep all equipment in the laminar flow cabinet while restreaking to avoid contamination. Once the inoculating loop leaves this area, discard it.

Features to look for to aid in identification of bacteria:

1. Colour
2. Texture / shape
3. Smell

DNA Extraction from a pure strain of bacteria

We conducted a DNA extraction and sequenced the 16S gene of the bacteria in order to identify the type of bacteria that we were working with. We wanted to know what bacteria was in the mixes that we created in order to do further research into their properties and identify the bacteria present in the different geographic areas around Rotorua, NZ.

*Equipment:*

* Bashing Bead Lysis Tube
* Lysis Solution
* Digital Cell Disruptor
* Microcentrifuge
* IV Spin Filter
* Collection tube
* DNA Pre-Wash Buffer
* IIC Column
* DNA Wash Buffer
* Microcentrifuge tube
* Distilled water
* Pipette
* Inoculation Loop

Note: the DNA extraction does not need to be carried out in a laminar flow cabinet (sterile environment).

*Method:*

1. Using an inoculation loop, add up to 0.25 grams of cultured bacteria from agar plates to a Bashing bead lysis tube (the bashing beads disrupt the biological sample: cell wall etc). Add 750µl Lysis solution to the tube. The Lysis solution stabilizes and preserves DNA.
2. Secure in bead beater fitted with a 2 ml tube holder assembly and process at maximum speed for 5 minutes.
3. Centrifuge the Bashing bead lysis tube in a Microcentrifuge at 10,000 x g for 1 minute. Centrifuging facilitates the separation of different phases extraction.
4. Transfer up to 400µl supernatant (a liquid formed in the chemical process of precipitation) using a pipette to a IV Spin filter in a collection tube and centrifuge at 7000 x g for 1 minute.
5. Add 1200µl of DNA binding buffer to the filtrate in the collection tube from step 4
6. Transfer 800µl of the mixture from step 5 to a Spin IIC column in a collection tube and centrifuge at 10000 x g for 1 minute.
7. Discard the flow through from the collection tube and repeat step 6
8. Add 500µl DNA wash buffer to the spin IIC column and centrifuge at 10000 x g for 1 minute (to wash DNA).
9. Transfer the spin IIC column to a clean 1.5ml Microcentrifuge tube and add 50µl of water directly to the column matrix. Centrifuge at 10000 x g for 30 seconds to elute (or resuspend) the DNA.
10. This produces PCR ready ultra pure DNA. Repeat this procedure for each sample of bacteria from which DNA is to be extracted.

16S Gene rDNA PCR (Polymerase Chain Reaction)  - Identification of Bacteria

Set up the following reactions to prepare the master mix

|  |  |  |  |
| --- | --- | --- | --- |
| Step | Reactions | X 1 | X 13 (µL) |
| A | ddH2O | 16.75 | 217.75 |
| B | PCR Buffer 10 x | 2.5 | 32.5 |
| C | dNTPs 2mM | 2.5 | 32.5 |
| D | 27F 5 µM (primer) | 1 | 13 |
| E | 1492R 5 µM (primer) | 1 | 13 |
| f | DNA | 0.5 |  |
| G | MgCl2 50 mM | 0.5 | 6.5 |
| H | Taq DNA pol  5U/µL Invitrogen | 0.25 | 3.25 |

Note: pipette up and down the reagents after each addition. Once the master mix is prepared, spin for 5 seconds in a microcentrifuge.

**Thermal Cycling:**

PCR amplification: Perform the PCR on a thermal cycler using the following program: (make sure to record the location of the samples)

|  |  |  |  |
| --- | --- | --- | --- |
| Step | Temperature (°C) | Time (minutes) | No. of Cycles |
| a1 | 94 | 3:00 | 1 |
| b2 | 94 | 1:00 | 25 |
| c3 | 55 | 1:00 | 25 |
| d4 | 72 | 1:00 | 25 |
| e5 | 72 | 7:00 | 1 |
| f6 (Hold) | 4 |  | 1 |

*nifH* Gene PCR - Nitrogen Fixing gene

Set up the following reactions to prepare the master mix

|  |  |  |  |
| --- | --- | --- | --- |
| Step | Reactions | X 1 | X 13 (µL) |
| A | ddH2O | 13.25 | 172.25 |
| B | PCR Buffer 10 x | 2.5 | 32.5 |
| C | dNTPs 2mM | 2.5 | 32.5 |
| D | *nifH*(polF) 5 µM (primer) | 2.5 | 32.5 |
| E | *nifH*(polR) 5 µM (primer) | 2.5 | 32.5 |
| f | DNA | 0.5 |  |
| G | MgCl2 50 mM | 1 | 6.5 |
| H | Taq DNA pol  5U/µL Invitrogen | 0.25 | 3.25 |

Note: pipette up and down the reagents after each addition. Once the master mix is prepared, spin for 5 seconds in a microcentrifuge.

**Thermal Cycling:**

PCR *nifH* amplification: Perform the PCR on a thermal cycler using the following program: (make sure to record the location of the samples).

|  |  |  |  |
| --- | --- | --- | --- |
| Step | Temperature (°C) | Time (minutes) | No. of Cycles |
| a1 | 94 | 3:00 | 1 |
| b2 | 94 | 1:00 | 30 |
| c3 | 55 | 1:00 | 30 |
| d4 | 72 | 0:30 | 30 |
| e5 | 72 | 5:00 | 1 |
| f6 | 4 | 24:00 | 1 |

**Gel Electrophoresis:**

*Equipment:*

- DNA solution (from bacterial samples)

- Agarose Gel

- Micropipette

- Buffer solution

- Loading Dye

- Fluorescent Dye

- Sub-Cell

- Power Supply

*Method:*

1. Prepare the agarose gel solution and pour into the gel chamber. Wait for it to solidify.
2. Align the wells so that they are closest to the negative electrode. DNA is negatively charged so when a current is run through the solution, the DNA runs down the gel, towards the positive electrode and is separated into its macromolecules.
3. Add the buffer solution into the cell wells until the solution covers the agarose gel.
4. Add the DNA samples with a loading dye (contains dye and glycerol).
5. Load the DNA samples (with loading dye), the positive/negative controls and the DNA ladder in the correct order according to the lanes/wells on the agarose gel they are to be run in.
6. Connect the electrodes to the power supply accordingly (black to black, red to red).
7. Set the voltage to 120V, and set the running time to 45 minutes.
8. Now the separation of the macromolecules on the agarose gel can be observed.
9. Suspend the agarose gel (with DNA samples run) in the fluorescent dye to allow for visualisation.
10. Repeat this procedure for the visualisation of the bacteria DNA,16S and *nifH* genes.

**Data analysis**

**ANOVA Tables:**

* + ***Radish Dry Weight (Bio Mass)***

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| SUMMARY |  |  |  |  |
| Groups | Count | Sum | Average | Variance |
| RW | 4 | 0.25 | 0.0625 | 0.001263 |
| KP | 4 | 0.216 | 0.054 | 0.000593 |
| US | 4 | 0.235 | 0.05875 | 0.000426 |
| KG | 3 | 0.105 | 0.035 | 7.90 x 10-5 |
| SM | 4 | 0.169 | 0.04225 | 0.000195 |
| SS | 4 | 0.282 | 0.0705 | 0.001577 |
| AV | 3 | 0.129 | 0.043 | 0.000427 |
| BS | 4 | 0.196 | 0.049 | 0.001599 |
| TU | 4 | 0.281 | 0.07025 | 0.00095 |
| SA | 4 | 0.212 | 0.053 | 0.000709 |
| NS | 4 | 0.39 | 0.0975 | 0.002199 |
| CN | 4 | 0.156 | 0.039 | 0.000176 |

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| ANOVA |  |  |  |  |  |  |
| Source of Variation | SS | df | MS | F | P-value | F crit |
| Between Groups | 0.012688 | 11 | 0.001153 | 1.304123 | 0.264251 | 2.083822 |
| Within Groups | 0.030071 | 34 | 0.000884 |  |  |  |
|  |  |  |  |  |  |  |
| Total | 0.042759 | 45 |  |  |  |  |

* + ***P. radiata Dry Weight (Bio Mass)***

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| SUMMARY | |  |  |  |
| Groups | Count | Sum | Average | Variance |
| RW | 4 | 0.316 | 0.079 | 0.000406 |
| KP | 4 | 0.2665 | 0.066625 | 0.00117 |
| US | 4 | 0.3329 | 0.083225 | 0.000361 |
| KG | 4 | 0.338 | 0.0845 | 0.001879 |
| SM | 4 | 0.357 | 0.08925 | 0.000416 |
| SS | 4 | 0.3761 | 0.094025 | 9.95E-05 |
| AV | 3 | 0.263 | 0.087667 | 0.000149 |
| BS | 4 | 0.372 | 0.093 | 0.004259 |
| TU | 4 | 0.345 | 0.08625 | 0.001051 |
| SA | 4 | 0.333 | 0.08325 | 0.000641 |
| NS | 3 | 0.294 | 0.098 | 0.000183 |

**Tabulation of Test Plant Dry Weights (Biomass):**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| ANOVA |  |  |  |  |  |  |  |
| Source of Variation | SS |  | df | MS | F | P-value | F crit |
| Between Groups | 0.002696 |  | 10 | 0.00027 | 0.265247 | 0.984492 | 2.153156 |
| Within Groups | 0.031508 |  | 31 | 0.001016 |  |  |  |
|  |  |  |  |  |  |  |  |
| Total | 0.034204 |  | 41 |  |  |  |  |

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Pine (g))** | **RW** | **KP** | **US** | **KG** | **SM** | **SS** | **AV** | **BS** | **TU** | **SA** | **NS** | **CN** |
| **1** | 0.105 | 0.047 | 0.087 | 0.082 | 0.071 | 0.092 | 0.077 | 0.041 | 0.048 | 0.111 | 0.111 |  |
| **2** | 0.057 | 0.106 | 0.0839 | 0.137 | 0.116 | 0.1061 | 0.085 | 0.099 | 0.098 | 0.082 | 0.099 | 0.05 |
| **3** | 0.082 | 0.083 | 0.104 | 0.088 | 0.076 | 0.082 | 0.101 | 0.049 | 0.124 | 0.050 | 0.084 |  |
| **4** | 0.072 | 0.0305 | 0.058 | 0.031 | 0.094 | 0.096 |  | 0.183 | 0.075 | 0.090 |  |  |

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Radishes (g)** | **RW** | **KP** | **US** | **KG** | **SM** | **SS** | **AV** | **BS** | **TU** | **SA** | **NS** | **CN** |
| **1** | 0.041 | 0.027 | 0.083 | 0.028 | 0.052 | 0.120 |  | 0.107 | 0.105 | 0.029 | 0.11 | 0.037 |
| **2** | 0.104 | 0.061 | 0.063 | 0.045 | 0.044 | 0.08 | 0.020 | 0.044 | 0.030 | 0.091 | 0.155 | 0.037 |
| **3** | 0.026 | 0.044 | 0.056 | 0.032 | 0.051 | 0.056 | 0.060 | 0.024 | 0.075 | 0.048 | 0.044 | 0.057 |
| **4** | 0.079 | 0.084 | 0.033 |  | 0.022 | 0.026 | 0.049 | 0.021 | 0.071 | 0.044 | 0.081 | 0.025 |

**Graphs of Test Plant Final Stem Height Growth:**

Note: Displayed here is a summary of the results. To see the full set of results refer to the appendix.

**Radishes**

Own treatments time series:

All trials treated with our mixes displayed similar trends in plant height. Between 5 June and 16 June, stem height decreased dramatically before a spike from 5 June to 29 June. Stem height did not increase to the initial height during this spike. This spike was almost non-existent in SM trials. By the next measurement, plants increased in height, with most trials reaching or exceeding initial height. This date was when plants in all own mix trials were at their peak stem height. The absolute max was in RW at approximately 3.25 cm. After this, plant height again decreased to less than or equal to initial height. Interestingly, at the last measurement on 25 July, heights across all trials seem to converge at about 2.5 cm.

Commercial treatments time series:

There is considerable variation among the trends of commercial treatments in the beginning of the time period, however by 29 June, they all follow a similar trend. Most commercial treatments decreased in height from time zero to 16 June, the same as our own treatments. The exceptions to this are AV and TU, both of which stayed relatively constant in their heights until 24 June. There is a peak for all other treatments between 5 June to 29 June. Because the initial drop was not as steep as that of our own mixes, the plants reach approximately their initial height in this peak. On the 9th July, all treatments were at maximum height before decreasing again at the end of the experiment. These final heights seem to converge around 3.25 cm, higher than the corresponding point in the own treatments graph.

Final stem height bar graph:

At the end of the experiment, most treatments were taller than the control. Exceptions are SS (slightly shorter), SA (on par with control) and KP and US (both more significantly shorter). US was shortest overall at 2.48 cm and KG tallest at 2.9 cm. The control was at 2.6 cm. It is interesting that our own treatments contained both the tallest and shortest. This may indicate more variability in the success of biofertilizers whereas traditional commercial fertilisers are more reliable or standardised.

One way ANOVA:

As the F value (1.3) is lower than the F critical value of 2.08, we fail to reject the null hypothesis that there is no difference between the means of the treatments groups.

Dry weight bar graph:

Although the ANOVA analysis proved that there was no statistically significant difference among the treatment groups, all except KG were heavier than the control. This is interesting as KG ended up being the tallest. The control was at 0.04 g, KG was at 0.036 g and NS (the heaviest) was at 0.096 g.

*P. radiata*

Own treatments time series (Stem height, cm):

Again all trials treated with our mixes displayed similar trends in plant stem height. Between 5 June and 16 June, stem height decreased dramatically before a spike from 5 June to 29 June. Stem height did not increase to initial height during this spike, with the exception of KP. By the next measurement, plants increased in stem height, with most trials reaching or exceeding initial height. This date was when plants in most own mix trials were at their peak stem height After this, plant stem height levels off, with the exception of CN, which we observe a sharp drop at 29 June, followed by an increase and later stem height levels off. The absolute max was in KP at 3.68 cm.

Commercial treatments time series (Stem height, cm):

Again there is considerable variation among the trends of commercial treatments in the beginning of the time period, however by 29 June, they all tend to follow a similar trend. Most commercial treatments decreased in stem height from time zero to 16 June. The exceptions to this are TU (also seen in Radishes). There is a peak for all other treatments between 5 June to 29 June. On the 25th July (final measurement), TU reaches its maximum height, while all other treatments on the 25th of July are almost all shorter than their initial heights, with the majority of peak stem heights being the initial height or height at 23rd June.

Final Stem height bar graph:

At the end of the experiment, most treatments had approximately the same stem height as the control. The maximum values being 3.68cm belonging to KP (own treatment) and TU (commercial), which are 0.45cm greater than the control. Exceptions are RW, AV and NZ (slightly shorter), US, SM, and SA (on par with control) and KP, KG, SS, BS and TU (taller than control). AV was shortest overall at 2.70 cm. The control was at 3.23 cm. Our treatments contained the fertilizer that produced the tallest stem height which is on par with the commercial TU treatment. Indicate that at least one of our treatments (KP) is on par with a commercial treatment (TU) in terms of *P. radiata* stem height (growth).

One way ANOVA:

As the F value (0.265) is lower than the F critical value of 2.153, we fail to reject the null hypothesis that there is no difference between the means of the treatments groups. (statistically insignificant).

Dry weight bar graph:

Although the ANOVA analysis proved that there was no statistically significant difference among the treatment groups, all treatments were heavier than the control.. The control was at 0.05 g. The heaviest was NS at 0.098g.

Mung beans

Own treatments time series (Stem height, cm):

RW, KP, US and CN display relatively little growth from the 5th June to the 29th June. With the exception of US, in which the stem height peaked at 11.80cm on 29th June. SM trials died first, by 15th June, with a shorter stem height than its initial height. The control’s stem height (CN) dropped significantly from 5th June to the 15th June, dropping approximately 1cm. It soon increased back to its initial height by the 23rd of June (high fluctuation). By the 29th of June 2016 all mung beans had died.

Commercial treatments time series (Stem height, cm):

The commercial treatments show little effect on the stem height. All treatments display a relatively constant stem height from the 5th of June to the 15th of June, at which point they slightly increase up to the 29th of June. At this point all of the mung beans have died. This is most likely due to environmental conditions (i.e. frost, cold temperatures).

Final Stem height bar graph:

After 24 days of growth only KP, US, SS, AV, BS and CN were alive (\*still dying). All treatments display a similar stem height to the control (CN), which is at 9.40cm. The longest stem height is AV (commercial) at 11.80cm with US on par (own treatment). However the lowest stem height value belongs to KP (own) at 9.35cm.

**Genetic analysis**

The DNA sequencing process found that *Nguyenibacter vanlangensis* lives in the soil of Kuirau Park and the Glenholme area in Rotorua, New Zealand. The sequence matched 100% (1345 bases/1345 base pairs) therefore there is high confidence that this organism was correctly identified. This bacterial species was first described in 2013 by Vu, Yukphan et al so additional information on the organism is limited. It was initially isolated on nitrogen-free media from a 2011 sample of Asian rice rhizosphere soil collected in Vietnam. Bacterium are observed to be rod shaped with flagella all over their surface. Colonies range from cream to brownish in colour and are smooth and transparent.

Furthermore DNA sequencing found that *Endobacter Medicaginis* lives in the soil of the Redwoods (RW1, RW2, and RW3) in Rotorua. The sequence matched  99% (1355/1357 base pairs). This displays a high confidence that the organism was correctly identified. This bacterial species was described by Ramirez-Bahena et al in 2013. This bacterial strain was first isolated from a surface-sterilized nodule of Medicago sativa in Zamora (Spain). This bacterial strain is a Gram-stain-negative, non-sporulating, aerobic coccoid to rod-shaped bacterium that was motile by a subpolar flagellum. It is a member of the Acetobacteraceae occurring as a legume nodule endophyte.

*Bacillus* species were isolated from Ngongotaha soils. This species is known to live in association with peanut plants and is capable of nitrogen fixation. (note: this bacteria was only recently discovered in 2013, published information is limited).

The *nifH* gene was not present in the Ngongotaha, Redwoods, Glenholme or Kuirau park isolated bacteria (excluding Utahina Stream and School Grounds – Utahina Area, as these bacterial samples did not show significant growth when conducting the DNA extraction so were unable to be analysed due to time constraints). This does not mean that the bacteria isolated were unable to fix nitrogen, but that they had other genes that were responsible for their nitrogen fixation function. (The bacteria collected were all able to fix nitrogen as they were able to grow in nitrogen limited conditions – see culturing in ‘Method’ section).

**Conclusion**

Our experiment does not fully support our hypothesis. Our predictions about the most effective commercial and own treatment have also been refuted (in terms of dry weights – not stem heights). This was determined by the One-Way ANOVA testing on the plant dry weights. The null hypothesis failed to be rejected, therefore our dry weight results are not statistically significant.

However in terms of stem height for *P. radiata* when the KP/ Kuirau Park (own) and TU/ Tui Urea (commercial) treatments were on par at 3.68cm (maximum stem height). For the radish stem height, KG/Glenholme, our treatment had the maximum stem height of 3.38cm. Therefore in terms of stem height for both the radishes and *P. radiata*, our treatments were on par or produced greater results than the commercial fertilizers. Furthermore the Kuirau Park treatment promoted the most growth (stem height – P. Radiata), which supports a statement in the hypothesis. Weaknesses in the experimental design will have been a factor affecting the accuracy and reliability of the results.

**Discussion**

**Experimental design**

The main strength of our experimental design was that we tested and compared a wide variety of treatments, both commercial and our own. None of the studies that we looked at (see ‘Comparisons to other studies’) evaluated their own biofertilizers alongside synthetic, commercially available fertilizers. So, we have definitely added an extra aspect to our project. However, this also came with increased time and labour costs. We quickly discovered this when applying the treatments at time zero. It took a long time to do this so we did not continue with our timetabled applications. This was not recommended use for the commercial fertilisers hence these trials may not have grown as well as they could have.

When applying the treatments to the plants, the commercial fertilizers had to be diluted as they were extremely concentrated (see calculations in logbook). For some products, this was difficult to do at home (for instance some fertilizers required only a few ml’s for a hectare) so we had to take some of the commercial fertilizers into the lab where we could accurately create the dilution necessary for our experiment.

24 days after the first treatments were applied, the mung beans died. This may be linked to a weakness in experimental design. As mung beans require a shorter growth period to reach maturity, the treatments may have been applied too late in development. Furthermore the mung beans were planted in winter (cold conditions). They are suited to grow in warm conditions and are susceptible to frost. Therefore the environmental conditions we kept the mung beans would have been outside their range of physiological tolerance. In order to prolong their growth they were covered with frost cloth and brought indoors (grew at room temperature), however this failed to prolong their growth.

**Accuracy improvement and error**

*Timing:* When applying treatments to the plants and measuring *P. radiata*, radish and mung bean stem heights, all measurements were taken within a 24 hour time frame. This allowed for the measurements of all the plants to be taken at the same stage of growth. All soil samples were collected within 24 hours, and had the same period of growth in the nitrogen limiting minimal media. This allows for all the mixes made to be at a similar concentration (microbial population), as the bacterial samples have all had the same time to grow in the media.

*Equipment accuracy:* By using microliter scale pipettes (piston-driven air displacement pipettes) this allowed for small volumes of liquids to be measured with great accuracy when conducting the serial dilution, bacterial culturing, DNA extraction, Polymerase Chain Reaction (PCR) and Agarose Gel Electrophoresis. Furthermore when weighing the dry weights, a scale that could measure values up to 4dp was used. This allowed for the accurate measurement of the dry weights of the plants. Therefore the values could be read to a greater number of significant figures and measurements were taken with greater accuracy.

*Zero error:* While measuring the test plant stem heights, in order to correct the zero error, the length of the ruler before the 0cm mark was measured and added to the total stem height. Furthermore when measuring the dry weight of the plants, the scales were set to zero after the container (that holds the plant) was placed on the scale. This avoided zero error, ensuring only the contents inside the container was weighed.

*Parallax error:* In order to avoid parallax error when measuring *P. radiata*, radish and mung bean stem heights, readings were taken directly in line with the measuring component (ruler). This allows the measurement taken to be an accurate reading - closer to true value.

*Repeated measurements:* In order to improve accuracy of the data four trials / repeats were conducted for each individual treatment for the *P. radiata*, mung beans and radishes. This increases the accuracy and reliability of the dry weights and plant stem heights. The averages were then taken, reducing the effect of random errors. Random errors will be either larger or smaller than the actual measurement causing a scattering around the true value. Through the process of repeating and averaging, the scattering is reduced, improving accuracy. Furthermore while culturing the bacteria, multiple restreaking and plating of the bacteria occurred. This allowed for a pure strain of a single colony of bacteria to be isolated and grown. Increasing the chances of correct identification of bacteria that is nitrogen fixing and used in this experiment (in the 16S gene and *nifH* gene PCR and sanger sequencing).

*Calibration error:* Calibration error was eliminated when measuring small volumes using microscale litre pipettes, centrifugation in DNA extraction (speed and time set), gel electrophoresis (apparatus voltage supply and time set), and thermal cycling (temperature, time and number of cycles set) by double checking and monitoring settings, programs and setting calibration on equipment. This is a type of systematic error, its effects cannot be reduced for example through repeating and averaging, as this error is consistently too high or too low.

**Comparisons to other studies**

*Islam et al (2009):*

Similar to us, the researchers of this study appear to have been working in the field of biofertilizers. The bacteria they used was isolated from rice fields. This is similar to our method of collecting samples from around Rotorua region. However, they found 17 diazotrophic strains, compared to our 3. They tested these bacteria on canola and rice plants which were different test plants to those in our study (*P. radiata*, radish and mung beans). The main point of difference between this study and ours is that this particular study found that most bacterial strains did significantly plant yield.

*Islam et al (2013):*

This study tested 11 bacterial strains on tomato and red pepper plants. This was different to the results of our study (found 3 bacterial strains – 2 unknown). This study also found statistically significant improvements in those plants that had been inoculated with the bacterial treatments.

*Indiragandhi, Anandham, Madhaiyan & Sa (2008):*

This study tested on canola and tomato plants, however the source of bacteria was quite novel. Eight strains were isolated from the guts of diamondback moths. Our mentor had initially suggested to us that we extract bacteria from huhu bugs but we chose not to follow through with this option due to ethical considerations. This study found that most strains had a significant positive effect on plant growth.

*Google Science Fair entry (Combating the global food crisis: diazotroph bacteria as a cereal crop growth promoter):*

The researchers of this project ran 9500 trials over 11 months so their experiment was on a much larger scale than our experiment. This study also began treatment from seed stage, whereas we waited until the test plants had germinated. Unlike our project, this study inoculated plants with varying concentrations of Rhizobium. In the interests of time, we relied on the assumption that the different soil conditions of the samples indicated different soil bacteria and this was not tested until the end of the project. This project also found significant increases in germination and growth of treated plants.

*Sarah L. Addison, Sonia M. Foote, Nicola M. Reid and Gareth Lloyd-Jones, 2007*

We see a similarity between our work in terms of certain experimental procedures. Both experiments use the isolation of the 16S gene and the *nifH* (350 bp) gene in a polymerase chain reaction. The 16S gene 1420 bp fragment was amplified using PCR (polymerase chain reaction) and sequenced to determine the genus and strain of the bacteria. Furthermore both experiments used a nitrogen-limited minimal medium (the same medium) to grow and isolate specific bacteria.

[*Hamilton TL*](http://www.ncbi.nlm.nih.gov/pubmed/?term=Hamilton%20TL%5BAuthor%5D&cauthor=true&cauthor_uid=21450003)*1,*[*Lange RK*](http://www.ncbi.nlm.nih.gov/pubmed/?term=Lange%20RK%5BAuthor%5D&cauthor=true&cauthor_uid=21450003)*,*[*Boyd ES*](http://www.ncbi.nlm.nih.gov/pubmed/?term=Boyd%20ES%5BAuthor%5D&cauthor=true&cauthor_uid=21450003)*,*[*Peters JW*](http://www.ncbi.nlm.nih.gov/pubmed/?term=Peters%20JW%5BAuthor%5D&cauthor=true&cauthor_uid=21450003)*., 2011 March, Yellow Stone National Park,* *BNF*

In this paper, biological nitrogen fixation in acidic high temperature geothermal springs (Yellowstone Nation Park, Wyoming) was investigated. In our experiment we took soil samples and isolated nitrogen fixing bacteria that was also located in an acidic high temperature geothermal area (Kuirau Park, Rotorua, NZ). Furthermore, both experiments isolated the *nifH* gene in the samples (sediments in Yellowstone/ soil samples in Kuirau Park).

**Recommendations for further research**

*Different plant species:* It would be interesting to observe how different plant species respond to fertiliser treatments. Although we found that there were no statistically significant differences between growth of the test plants (in terms of dry weight only after treatments were applied), other plant species may respond more positively to certain treatments. This would allow us to find the fertiliser that works best for a certain plant type. A possible application of this would be plant specific fertilisation. That is, growth of the desired plant would be encouraged while other plants, such as weeds, would experience insignificant change. Some plant species that would be beneficial to test are important New Zealand exports such as kiwifruit.

*Longer growth period:* Due to time constraints on project completion, our actual experiment (plant growth) ran for a relatively short amount of time (approx. 2 months) so we are unable to confidently predict long term effects of fertiliser treatment on plant growth. Most treatments displayed a slight increase in plant height over the last two weeks of the trial. Continuation of this trend in our own treatments would indicate that our mixes are feasible for wider use. Time constraints also affected some other aspects of our experiment. For example, we were unable to conduct genetic analysis on bacteria from all mixes (Mt Ngongotaha and JPC – Utahina were not tested) as these cultures did not show sufficient growth to get a relatively large quality to extract DNA from.

*Different bacterial mixes:* All our own mixes originated from the local Rotorua area, therefore there was not that much variation in soil types and bacteria in our collected samples. Samples from further afield would have included different bacteria that may have been more effective at forming symbioses with our test plant species. This would have lead to better growth of test plants and a more effective mix / biofertilizer. As a result of this, we could have discovered that certain soil bacteria are best suited for association with particular plants. An application of this is that decisions regarding planting crops could be better informed after taking into account the soil bacteria likely to live in the soil of the particular area. We considered the option of purchasing bacteria however ultimately decided against this due to constraints of our budget.

*Different treatment concentrations:* Experimenting with different concentrations of each treatment would have helped us to find which concentration maximises plant growth, allowing more economical use of fertilisers. The commercial fertilisers were probably already used at this concentration as the manufacturers would have already done testing to determine the specific concentration, and provided it as the recommended use guide. However we could not do this with our own mixes as we did not know what concentration they were at and had limited time to test this.

**Application of findings**

Biofertilizers containing nitrogen fixing bacteria have real world implications that can facilitate plant growth, biomass and overall soil health. Nitrogen fixing bacteria reduce crops’ water requirements, as the organisms release a gel-like substance which serves as a protective bio-film in order to slow down predators. This substance retains moisture in the root zone and leads to significant reduction in irrigation requirements (with the introduction of the nitrogen fixing bacteria in soil). In addition, the use of nitrogen fixing bacteria builds microbial populations and biodiversity within the soil, aiding in disease suppression in the soil and encouraging soil food-web balance (Islam, R et al).

In nitrogen fixing bacteria the enzyme complex nitrogenase catalyses the reaction by which N2 is converted into ammonia. Nitrogenase activity is strictly controlled in order to optimise the metabolism of the bacteria. For instance, the enzyme is inactivated by oxygen and excess nitrogenous compounds in the soil. Controlling and maintaining the balance of nitrogenous compounds in the soil, so that only the plant’s requirements are met. This means excess ammonia is not produced in order to optimise the bacteria’s metabolism, reducing nitrogen expenditure. Furthermore, nitrogen fixing bacteria produce nitrogen over extended periods of time unlike conventional nitrogen inputs (Horrigan, L. et at), due to the constant reproduction of the bacteria and establishment of the population within the soil root zone.

The amount of nitrogen compounds in soil is a factor affecting nitrogen leaching. Nitrate is susceptible to transformation and leaching. Therefore, through the use of nitrogen fixing bacteria would decrease the concentration of nitrates and maintain a balance of nitrogen in soil. Reducing the effects of nitrogen leaching, and the amount of nutrients (specifically nitrates) entering streams, lakes and other bodies of water, consequently, slowing the process of anthropogenic eutrophication. This will increase water quality, reduce non-point loading of nutrients, increase and maintain biodiversity in marine environments, and reduce costs in removal of weeds (see algae proliferation), water quality and waste management / treatment.

Based on our research, we propose a biofertilizer containing a dual strain formulation of *Nguyenibacter vanlangensis* and *Bacillus megaterium* bacteria. These bacteria fix nitrogen in symbiosis with plants and also mobilize essential elements such as phosphorus to make them available for plant use. It would be a superior biological alternative to synthetic fertilizers currently used as it is cheaper, more efficient in nutrient delivery, has additional soil-enriching benefits, and has no harmful environmental impacts.

*N. vanlangensis* and *B. megaterium* solubilize phosphate[[1]](#endnote-1) through the release of organic acids (including gluconic, citric, lactic, propionic, succinic acids). Their hydroxyl and carboxyl groups chelate phosphate cations, converting the phosphate into a soluble form available for plant utilization. *Bacillus megaterium* is also able to mobilize potassium (K) in the soil. Potassium is typically abundant in soil, however 98% of potassium is bound in mineral forms, unavailable for plant use. K aids in ATP energy production, water and nutrient transportation in plants. *B. megaterium* mobilizes potassium through the production of organic acids (formic, malic and oxalic acids), which supply protons, enhancing the dissolution of potassium compounds (potash). Solubilization occurs by the complex formation between organic acids and metal ions (e.g. Fe2+, Al3+, Ca2+). Lastly, *B. megaterium* also solubilizes zinc (Zn) through the production of organic acids, which converts insoluble zinc compounds by lowering soil pH. In plants, zinc is a key constituent in enzymes and proteins and plays an important role in hormone production and internode elongation.

There are a number of biofertilizer patents that use different bacterial strain formulations. These bacteria use the same mechanisms of nitrogen fixation/element solubilisation and mobilization as our proposed formulation. However, due to the specific bacteria used, most of these existing biofertilizers only provide one or two plant nutrients. This is unlike our formulation which would provide nitrogen, phosphorus, and potassium, as well as trace elements.

At the moment, our biofertilizer mix is an unoptimized prototype however we are confident that with further research and field trials, we will be able to refine our formula and offer an alternative to synthetic fertilizers that performs just as well, if not better, in terms of promoting plant growth and production.

**Appendix A: GLOBE badge descriptions**

**Collaboration**

**It was incredibly helpful to have both of us working together on this project. Our individual strengths and personalities complement and balance each other so as a team we are well-rounded. Our friendship also aids our team dynamics. We understand each other so work well together and can discuss things openly and trust one another.** All members of our team were invested in participating to the best of their ability and contributed equally to the project. **By combining our knowledge, resources, skills, and time, we were able to complete a more comprehensive project.**

Shulan Qiu constantly challenged and motivated the team to work harder. Her timelines and lists helped us organize and prioritize tasks that needed to be done. She also set up the Google Docs we used throughout our project. Whenever we worked in the laboratory or met with our mentor, Shulan took detailed notes for us to refer back to later on. In addition, she managed project finances and tracked expenses to make sure we didn’t go over budget. Without her determination and pep talks, nothing would have gotten done.

Kesia Kurian communicated with relevant organisations and people on behalf of the team. Her emails to local businesses and fertilizer manufacturers allowed us to secure free samples of commercial products to test. We also had the opportunity to connect with a STEM professional through her work experience placement at Scion (Forest Research Institute in Rotorua). Kesia also ran the ANOVA data analysis tests. She is detail oriented and ensured that the team and its work met all criteria.

One advantage of collaborating on this project was that we were able to use each other as sounding boards when discussing potential ideas. Having to explain ourselves to each other was a real test of how well we understood the topic and the process often revealed possible issues before they actually arose. This allowed us to use time efficiently and focus our efforts on only the most promising ideas. Similarly, if one of us did not understand something about the laboratory technique or the literature, we were able to discuss the matter with the other person. Often, the matter would be cleared up amongst ourselves and we would not have to look elsewhere.

We worked on this project for the better part of a year and at times, our motivation dipped and we felt overwhelmed and frustrated. During these times, it was good to know that there was someone working alongside us. The shared responsibility for the project meant that we did not want to let each other down and this helped increase morale again.

**Connecting with STEM professional**

**We were incredibly fortunate to have Sarah Addison, an experienced microbiologist, mentoring us throughout all phases of the project. Her involvement was invaluable in taking our project to a more sophisticated level. Later on in the project, Beccy Ganley, a forest pathologist, helped us develop our research into a feasible engineering solution.**

**Initially, with Sarah’s guidance we developed specific research questions and decided to develop our own biofertilizer mixes in addition to trialling existing products. This added another dimension to our project and made it more relevant to the real world. When we were conducting the literature review, she helped us access some papers. This was particularly helpful as we found that full access to a lot of papers had to be bought and was quite expensive. She also looked over our report once completed and gave us some pointers on how to make it meet the typical requirements of a scientific paper (e.g. writing species names correctly). We feel that this made our final outcome more polished.**

**Perhaps the most valuable thing that came from our collaboration with Sarah was the incorporation of specialised scientific equipment and techniques into our project. Sarah and her employer, Scion, were generous enough to let us come into her labspace and conduct our experiments. This meant that we were able to analyse nitrogen fixing bacteria from our local environment, as opposed to using bacterial samples purchased from elsewhere. Our school does not have the equipment to do genetic analysis so in this way, collaboration with Sarah, a STEM professional, enhanced our research methods. The depth of our project also improved as a result of this collaboration because we were able to hypothesise links between the soil types and the bacteria that we identified.**

**The experience of working in a professional laboratory environment was significant for both of us. We got to practice skills such as streaking and pipetting and were able to see the theory we had read about in action. Going forward, this experience has encouraged us to undertake similar projects in future, and has affirmed our decisions to pursue STEM careers.**

**Engineering solution**

**We have applied our research and developed a concept for a biofertilizer containing diazotrophs as a possible solution to the issue of nutrient loss from agricultural practices (see ‘Application of findings’). Beccy Ganley helped us with this part of the project.**

**We wanted our proposed formulation to bring together the best of existing fertilizer types (cheap, effective) while mitigating their adverse effects (adverse environmental impacts). For instance, organic fertilizers are low impact on the environment but have low nutrient density. Synthetic fertilizers are relatively cheap and easy to produce however are not very efficient in terms of nutrient delivery to plants (**on average, crops only recover 40%-65% of nitrogen, 15%-25% of phosphorus, and 30%-50% of potassium from synthetic fertilizers[[2]](#endnote-2)). Existing biofertilizers are more competitive in terms of nutrient delivery and do not impact the environment but as they are an emerging technology, they tend to be expensive and have limited application windows.

If our optimized formulation (or other biofertilizers like it) are used worldwide in place of synthetic fertilizers, the impact would be enormous. This would be a step towards organic agriculture which will change the way that food is grown on earth. It would improve plant productivity in a sustainable manner for the long term, ensuring food security for the increasing global population both today and in future. With nutrient loss into the environment being reduced by this change, it could be expected that the affected ecosystems would rebalance.

The primary criteria that we decided our proposed biofertilizer had to meet was a low environmental impact. We feel we have achieved this in terms of minimal nutrient loss however, the impacts of introducing microorganisms into different soil types would have to be looked into. Furthermore, in order to be a practical, feasible solution, our biofertilizer’s nutrient delivery would have to be on par, if not better than, that of synthetic biofertilizers. As discussed previously, in the ‘Applications’ section of this report, more refinement and research is needed in this area for this criteria to be met.

**Appendix B: Additional data and graphs**

**Test Plant Heights – Measurements**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| ***P. radiata* (cm)** | **5 June** | **16 June** | **23 June** | **29 June** | **10 July** | **25 July** |
|  |  |  |  |  |  |  |
| RW1 | 3.3 | 2.9 | 3.1 | No measurement | 3.2 | 3.1 |
| RW2 | 3.0 | 2.0 | 2.1 | 2.6 | 2.7 | 2.7 |
| RW3 | 3.1 | 2.6 | 2.9 | 2.5 | 2.7 | 3.3 |
| RW4 | 3.4 | 2.6 | 2.8 | 3.3 | 2.9 | 3.3 |
| **RW Avg** | **3.2** | **2.5** | **2.7** | **2.8** | **2.9** | **3.1** |
|  |  |  |  |  |  |  |
| KP1 | 2.6 | 2.4 | 2.7 | No measurement | 2.7 | 2.8 |
| KP2 | 5.4 | 4.7 | 5.1 | 5.5 | 5.5 | 5.7 |
| KP3 | 3.6 | 3.9 | 3.9 | 3.1 | 3.9 | 3.6 |
| KP4 | 2.2 | 1.9 | 2.4 | 2.4 | 2.5 | 2.6 |
| **KP Avg** | **3.6** | **3.2** | **3.5** | **3.7** | **3.7** | **3.7** |
|  |  |  |  |  |  |  |
| US1 | 3.2 | 3.0 | 3.0 | 2.8 | 3.4 | 3.1 |
| US2 | 3.5 | 3.2 | 3.0 | 3.1 | 3.4 | 3.1 |
| US3 | 3.4 | 2.9 | 3.1 | 2.7 | 2.7 | 3.1 |
| US4 | 3.2 | 2.3 | 3.2 | 3.2 | 3.4 | 3.6 |
| **US Avg** | **3.3** | **2.9** | **3.1** | **3.0** | **3.2** | **3.2** |
|  |  |  |  |  |  |  |
| KG1 | 3.1 | 2.4 | 3.0 | 3.3 | 3.2 | 3.3 |
| KG2 | 3.2 | 2.3 | 2.9 | 3.0 | 2.9 | 3.3 |
| KG3 | 3.7 | 3.0 | 3.6 | 3.5 | 3.9 | 3.9 |
| KG4 | 3.8 | 2.8 | 3.6 | 3.6 | 3.6 | 3.7 |
| **KG Avg** | **3.5** | **2.6** | **3.3** | **3.4** | **3.4** | **3.6** |
|  |  |  |  |  |  |  |
| SM1 | 3.9 | 2.7 | 3.6 | 3.4 | 3.4 | 3.9 |
| SM2 | 2.4 | 2.0 | 2.1 | 2.5 | 2.0 | 2.1 |
| SM3 | 3.8 | No measurement | 3.6 | 3.9 | 3.9 | 4.0 |
| SM4 | 2.8 | 2.9 | 3.1 | 3.9 | 3.4 | 2.9 |
| **SM Avg** | **3.3** | **2.5** | **3.1** | **3.4** | **3.2** | **3.2** |
|  |  |  |  |  |  |  |
| SS1 | 3.9 | 3.8 | 4.2 | 3.6 | 4.2 | 3.9 |
| SS2 | 3.7 | 3.4 | 3.7 | 3.3 | 3.6 | 3.4 |
| SS3 | 3.2 | 2.0 | 2.9 | 2.7 | 2.9 | 3.3 |
| SS4 | 3.3 | 2.3 | 2.9 | 3.1 | 3.1 | 3.2 |
| **SS Avg** | **3.5** | **2.9** | **3.4** | **3.2** | **3.5** | **3.5** |
|  |  |  |  |  |  |  |
| AV1 | 2.7 | 1.9 | 2.3 | 2.3 | 2.7 | 2.7 |
| AV2 | 2.8 | 2.4 | 2.8 | 1.7 | 2.7 | 2.5 |
| AV3 | 3.4 | 2.8 | 2.6 | 2.4 | 3.4 | 2.9 |
| **AV Avg** | **3.0** | **2.4** | **2.6** | **2.1** | **2.9** | **2.7** |
|  |  |  |  |  |  |  |
| BS1 | 4.5 | 3.7 | 4.3 | 3.2 | 4.3 | 4.3 |
| BS2 | 3.5 | 3.3 | 3.5 | 4.3 | 3.4 | 3.3 |
| BS3 | 3.1 | 2.9 | 2.5 | 2.2 | 3.1 | 2.8 |
| BS4 | 3.5 | 2.6 | 3.0 | 3.2 | 3.4 | 3.4 |
| **BS Avg** | **3.7** | **3.1** | **3.3** | **3.2** | **3.6** | **3.5** |
|  |  |  |  |  |  |  |
| TU1 | 2.7 | 2.1 | 2.4 | 2.9 | 2.7 | 2.6 |
| TU2 | 3.4 | 3.0 | 3.5 | 2.5 | 3.3 | 3.4 |
| TU3 | 2.9 | 4.2 | 4.3 | 4.5 | 4.3 | 4.8 |
| TU4 | 3.9 | 4.0 | 4.1 | 3.3 | 3.9 | 3.9 |
| **TU Avg** | **3.2** | **3.3** | **3.6** | **3.3** | **3.6** | **3.7** |
|  |  |  |  |  |  |  |
| SA1 | 3.1 | 2.4 | 3.0 | 2.8 | 2.9 | 3.3 |
| SA2 | 3.5 | 3.6 | 3.3 | 3.0 | 3.3 | 3.6 |
| SA3 | 2.5 | No measurement | 2.4 | 2.2 | 2.4 | 2.5 |
| SA4 | 3.5 | 3.1 | 3.5 | 3.1 | 3.6 | 3.5 |
| **SA Avg** | **3.2** | **3.0** | **3.1** | **2.8** | **3.1** | **3.2** |
|  |  |  |  |  |  |  |
| NS1 | 3.7 | 3.1 | 3.6 | 3.1 | 3.6 | 3.9 |
| NS2 | 2.9 | 2.5 | 2.5 | 2.2 | 2.6 | 2.7 |
| NS3 | 2.7 | 3.2 | 2.8 | 2.3 | 2.8 | 2.8 |
| **NS Avg** | **3.1** | **2.9** | **3.0** | **2.5** | **3.0** | **3.1** |
|  |  |  |  |  |  |  |
| CN1 | 3.5 | 3.1 | 3.3 | 2.7 | 3.5 | 3.3 |
| CN2 | 3.2 | 2.4 | 2.9 | 2.5 | 2.9 | 3.0 |
| CN3 | 3.2 | 2.7 | 3.2 | 3.2 | 3.4 | 3.4 |
| **CN Avg** | **3.3** | **2.7** | **3.1** | **2.8** | **3.3** | **3.2** |

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Radishes (cm)** | **5 June** | **16 June** | **24 June** | **29 June** | **10 July** | **25 July** |
|  |  |  |  |  |  |  |
| RW1 | 3.5 | 3.2 | 3.5 | 2.3 | 3.5 | 3.4 |
| RW2 | 3.2 | 2.2 | 2.6 | 2.6 | 2.9 | 2.4 |
| RW3 | 3.5 | 2.1 | 2.7 | 2.4 | 3.2 | 2.9 |
| RW4 | 2.5 | 2.3 | 2.2 | 2.6 | 3.5 | 1.9 |
| **RW Avg** | **3.2** | **2.5** | **2.8** | **2.5** | **3.3** | **2.7** |
|  |  |  |  |  |  |  |
| KP1 | 2.6 | 1.6 | 2.4 | 2.3 | 2.4 | 2.4 |
| KP2 | 2.8 | 2.6 | 2.9 | 2.7 | 3.4 | 2.7 |
| KP3 | 2.2 | 1.9 | 1.9 | 1.8 | 3.4 | 2 |
| KP4 | 2.1 | 2.7 | 2.4 | 2.4 | 2.9 | 3.0 |
| **KP Avg** | **2.4** | **2.2** | **2.4** | **2.3** | **3.0** | **2.5** |
|  |  |  |  |  |  |  |
| US1 | 3.2 | 2.5 | 2.7 | 2.9 | 3.1 | 2.7 |
| US2 | 3.1 | 2.0 | 2.5 | 1.7 | 2.7 | 3.0 |
| US3 | 2.5 | 2.3 | 2.1 | 1.7 | 2.7 | 1.9 |
| US4 | 2.8 | 2.8 | 2.8 | 3.0 | 3.5 | 2.3 |
| **US Avg** | **2.9** | **2.4** | **2.5** | **2.3** | **3.0** | **2.5** |
|  |  |  |  |  |  |  |
| KG1 | 2.7 | 2.2 | 2.7 | 2.3 | 2.9 | 2.7 |
| KG2 | 3.9 | 2.6 | 3.8 | 3.2 | 3.8 | 3.5 |
| KG3 | 3.6 | 3.5 | 3.2 | 3.1 | 3.4 | 2.9 |
| KG4 | 2.4 | 2.2 | 2.2 | 2.4 | 3.4 | 2.5 |
| **KG Avg** | **3.2** | **2.6** | **3.0** | **2.8** | **3.4** | **2.9** |
|  |  |  |  |  |  |  |
| SM1 | 3.8 | 3.2 | 3.1 | 3.0 | 4.0 | 3.0 |
| SM2 | 3.1 | 3.0 | 2.9 | 2.9 | 3.6 | 2.9 |
| SM3 | 2.8 | 2.5 | 2.6 | 2.0 | 2.9 | 2.5 |
| SM4 | 2.7 | 2.1 | 2.4 | 2.7 | 2.9 | 2.8 |
| **SM Avg** | **3.1** | **2.7** | **2.9** | **2.7** | **3.4** | **2.8** |
|  |  |  |  |  |  |  |
| SS1 | 2.3 | 2.0 | 1.8 | 2.2 | 2.8 | 2.4 |
| SS2 | 2.9 | 2.5 | 2.9 | 2.7 | 3.4 | 3.1 |
| SS3 | 2.5 | 2.6 | 2.5 | 2.5 | 3.2 | 2.5 |
| SS4 | 3.4 | 2.3 | 2.4 | 2.3 | 2.8 | 2.3 |
| **SS Avg** | **2.8** | **2.4** | **2.4** | **2.4** | **3.1** | **2.6** |
|  |  |  |  |  |  |  |
| AV1 | 2.8 | 3.1 | 3.0 | 2.9 | 3.3 | 2.6 |
| AV2 | 4.0 | 3.4 | 4.0 | 3.6 | 4.0 | 3.7 |
| AV3 | 3.1 | 3.1 | 3.0 | 2.7 | 3.1 | 2.7 |
| AV4 | 2.1 | 2.7 | 2.0 | 1.6 | 2.4 | 2.3 |
| **AV Avg** | **3.0** | **3.1** | **3.0** | **2.7** | **3.2** | **2.8** |
|  |  |  |  |  |  |  |
| BS1 | 3.4 | 2.1 | 2.9 | 2.2 | 3.0 | 2.8 |
| BS2 | 3.7 | 3.5 | 3.7 | 3.8 | 3.9 | 3.6 |
| BS3 | 2.7 | 2.2 | 2.6 | 2.3 | 2.9 | 2.3 |
| BS4 | 2.2 | 2.0 | 2.0 | 1.9 | 2.7 | 2.4 |
| **BS Avg** | **3.0** | **2.5** | **2.8** | **2.6** | **3.1** | **2.8** |
|  |  |  |  |  |  |  |
| TU1 | 2.7 | 2.4 | 2.7 | 2.7 | 3.4 | 2.6 |
| TU2 | 2.8 | 3.0 | 2.5 | 2.9 | 2.5 | 2.3 |
| TU3 | 2.0 | 2.3 | 2.3 | 2.0 | 2.9 | 2.8 |
| TU4 | 3.8 | 3.4 | 3.6 | 3.3 | 3.8 | 3.5 |
| **TU Avg** | **2.8** | **2.8** | **2.8** | **2.7** | **3.2** | **2.8** |
|  |  |  |  |  |  |  |
| SA1 | 2.7 | 2.5 | 2.4 | 2.7 | 3.1 |  |
| SA2 | 2.5 | 1.1 | 1.9 | 1.7 | 2.4 | 2.6 |
| SA3 | 2.8 | 3.1 | 2.5 | 1.9 | 2.5 | 2.4 |
| SA4 | 3.1 | No measurement | 3.0 | No measurement | 2.9 | 2.8 |
| **SA Avg** | **2.8** | **2.2** | **2.5** | **2.1** | **2.7** | **2.6** |
|  |  |  |  |  |  |  |
| NS1 | 2.6 | 2.4 | 2.4 | 2.5 | 3.2 | 2.5 |
| NS2 | 3.5 | 3.1 | 3.3 | 2.9 | 3.2 | 3.3 |
| NS3 | 2.0 | 2.6 | 2.7 | 2.8 | 3.4 | 2.9 |
| NS4 | 2.5 | 1.9 | 2.6 | 2.0 | 2.9 | 2.7 |
| **NS Avg** | **2.7** | **2.5** | **2.8** | **2.6** | **3.2** | **2.9** |
|  |  |  |  |  |  |  |
| CN1 | 2.7 | 2.4 | 2.6 | 2.8 | 2.9 | 2.8 |
| CN2 | 3.1 | 2.1 | 2.7 | 2.3 | 2.8 | 2.5 |
| CN3 | 2.4 | 2.2 | 2.4 | 2.0 | 2.7 | 2.4 |
| CN4 | 2.8 | 2.1 | 2.3 | 2.2 | 2.9 | 2.7 |
| **CN Avg** | **2.8** | **2.2** | **2.5** | **2.3** | **2.8** | **2.6** |
|  |  |  |  |  |  |  |
| For second measurement, leaf length was taken so we will not be including 16 June data points in our graphs. | | | | | | |

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Mung Bean (cm)** | **5 June** | **15 June** | **23 June** | **29 June** |
|  |  |  |  |  |
| RW1 | 10.7 | 9.9 | 10.2 |  |
| RW2 | 10.5 | 11 | 10.5 |  |
| RW3 | 9.70 | 9 |  |  |
| RW4 | 10.0 | 9.4 |  |  |
| **RW Avg** | **10.2** | **9.8** | **10.4** |  |
|  |  |  |  |  |
| KP1 | 10.8 | 10.4 | 10.6 | 10.9 |
| KP2 | 9.9 | 9.8 |  |  |
| KP3 | 7.6 | 7.5 | 8.0 | 7.8 |
| KP4 | 11.3 | 10.5 | 10.3 |  |
| **KP Avg** | **9.9** | **9.6** | **9.2** | **9.4** |
|  |  |  |  |  |
| US1 | 9.00 | 9.20 | 9.10 |  |
| US2 | 11.2 | 11.0 | 12.2 |  |
| US3 | 10.0 | 10.0 | 10.1 |  |
| US4 | 12.4 | 11.9 | 11.9 | 11.8 |
| **US Avg** | **10.7** | **10.5** | **10.8** | **11.8** |
|  |  |  |  |  |
| KG1 | 8.3 | 8.1 |  |  |
| KG2 | 9.3 | 8.4 |  |  |
| KG3 | 10.6 | 10.3 | 9.7 |  |
| KG4 | 8.2 | 7.9 |  |  |
| **KG Avg** | **9.1** | **8.7** | **9.7** |  |
|  |  |  |  |  |
| SM1 | 9.1 | 9.5 |  |  |
| SM2 | 8.2 | 7.6 |  |  |
| SM3 | 11.0 | 10.9 |  |  |
| SM4 | 7.9 | 7.4 |  |  |
| **SM Avg** | **9.1** | **8.9** |  |  |
|  |  |  |  |  |
| SS1 | 8.70 | 7.80 |  |  |
| SS2 | 10.3 | 11.4 |  |  |
| SS3 | 11.2 | 11.2 | 11.2 | 11.1 |
| SS4 | 11.6 | 11.8 |  |  |
| **SS Avg** | **10.5** | **10.6** | **11.2** | **11.1** |
|  |  |  |  |  |
| AV1 | 8.00 | 7.80 |  |  |
| AV2 | 9.60 | 9.10 | 9.80 |  |
| AV3 | 11.0 | 11.3 | 11.1 | 11.2 |
| AV4 | 11.9 | 12.1 | 12.5 | 12.4 |
| **AV Avg** | **10.1** | **10.1** | **11.1** | **11.8** |
|  |  |  |  |  |
| BS1 | 9.5 | 9.4 | 9.8 | 9.8 |
| BS2 | 9.7 | 9.2 | 9.6 | 9.7 |
| BS3 | 10.5 | 11.6 |  |  |
| BS4 | 9.6 | 9.4 | 9.5 |  |
| **BS Avg** | **9.8** | **9.9** | **9.6** | **9.8** |
|  |  |  |  |  |
| TU1 | 9.7 | 9.8 | 9.5 |  |
| TU2 | 6.7 | 6.5 |  |  |
| TU3 | 8.8 | 9.2 |  |  |
| TU4 | 11.5 | 11.5 | 12.0 |  |
| **TU Avg** | **9.2** | **9.3** | **10.8** |  |
|  |  |  |  |  |
| SA1 | 11.1 | 11.1 |  |  |
| SA2 | 9.2 | 8.7 |  |  |
| SA3 | 8.0 | 7.3 |  |  |
| SA4 | 9.7 | 9.3 |  |  |
| **SA Avg** | **9.5** | **9.1** |  |  |
|  |  |  |  |  |
| NS1 | 11.7 | 11.2 | 11.1 |  |
| NS2 | 9.1 | 9.3 | 9.0 |  |
| NS3 | 11.6 | 10.7 | 11.1 |  |
| NS4 | 10.5 | 10.1 | 10.4 |  |
| **NS Avg** | **10.7** | **10.3** | **10.4** |  |
|  |  |  |  |  |
| CN1 | 10.8 | No measurement |  |  |
| CN2 | 10.5 | 10.1 | 10.3 |  |
| CN3 | 8.6 | 8.8 |  |  |
| CN4 | 9.2 | 8.9 | 9.3 | 9.4 |
| **CN Avg** | **9.8** | **9.3** | **9.8** | **9.4** |

**Test Plant Dry Weights (Biomass)**

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Pine (g)** | **RW** | **KP** | **US** | **KG** | **SM** | **SS** | **AV** | **BS** | **TU** | **SA** | **NS** | **CN** |
| **1** | 0.105 | 0.047 | 0.087 | 0.082 | 0.071 | 0.092 | 0.077 | 0.041 | 0.048 | 0.111 | 0.111 |  |
| **2** | 0.057 | 0.106 | 0.0839 | 0.137 | 0.116 | 0.1061 | 0.085 | 0.099 | 0.098 | 0.082 | 0.099 | 0.05 |
| **3** | 0.082 | 0.083 | 0.104 | 0.088 | 0.076 | 0.082 | 0.101 | 0.049 | 0.124 | 0.050 | 0.084 |  |
| **4** | 0.072 | 0.0305 | 0.058 | 0.031 | 0.094 | 0.096 |  | 0.183 | 0.075 | 0.090 |  |  |

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Radishes (g)** | **RW** | **KP** | **US** | **KG** | **SM** | **SS** | **AV** | **BS** | **TU** | **SA** | **NS** | **CN** |
| **1** | 0.041 | 0.027 | 0.083 | 0.028 | 0.052 | 0.120 |  | 0.107 | 0.105 | 0.029 | 0.11 | 0.037 |
| **2** | 0.104 | 0.061 | 0.063 | 0.045 | 0.044 | 0.08 | 0.020 | 0.044 | 0.030 | 0.091 | 0.155 | 0.037 |
| **3** | 0.026 | 0.044 | 0.056 | 0.032 | 0.051 | 0.056 | 0.060 | 0.024 | 0.075 | 0.048 | 0.044 | 0.057 |
| **4** | 0.079 | 0.084 | 0.033 |  | 0.022 | 0.026 | 0.049 | 0.021 | 0.071 | 0.044 | 0.081 | 0.025 |

*Note: Mung Bean dry weights are not included as the plants died after 24 days.*

**Acknowledgements:**

This project would not have been possible without the input of many others. We would like to take this opportunity to formally thank them for so generously supporting us.

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