university of groningen

Exploring the exclusion of Bacillus mycoides in plants from the Legume family

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Abstract

A healthy soil microbiome is a key factor to consider for world food production as specific bacteria are beneficial to plant growth. Finding a new bacterial species to add to soil could mean better agricultural yields. One contender is *Bacillus mycoides*, an endophytic strain of bacteria capable of nitrogen fixation. However, while able to endo-colonize different plant species, *B. mycoides* M2E15 was found to be excluded from members of the legume family. Testing the hypothesis that the capacity to endocolonize depends on the growth conditions of the plant, plants from the legume family were subjected to abiotic stress before inoculation. Although no effect of inoculation on plant growth was found, there was an effect on root nodule forming. Although the size of nodules differed between *B. mycoides* treatments, no effect on nitrogen availability was seen, nor an effect on the growth and photosynthetic capability of legume plants.

Introduction

The rhizosphere surrounding plant roots naturally harbor many distinct species of bacteria and other microorganisms. While some, like the nitrogen-fixing rhizobia, can be beneficial to plant species there are also many plant-associated bacteria that are harmful to plant health (Pini *et al*., 2012). To mitigate these harmful effects of bacteria, plants release upwards of 20 percent of their photosynthesis products into the soil. These so-called root exudates play different roles in root functioning, some of the components of root exudate are used to establish plant-microorganism interactions while some are used to inhibit soil-borne pathogens (Haichar *et al*., 2008). Some of these plant-associated bacteria, like some species of Rhizobium, have been studied in great detail. The reason for this is the value of these bacteria in biological fertilization. One of these species is *Rhizobium tropici* which was used by researchers from Brazil as a candidate for biological fertilization in *Phaseolus vulgaris* (French Bean). This species has been grown in Brazil for many years and is one of the most important sources of protein and carbohydrates in the country. *Phaseolus vulgaris,* like any other plant, needs nitrogen as a building block for growth and for the overall functioning of the plant. The use of nitrogen fixing bacteria, in this case *Rhizobium tropici*, would significantly decrease the need for nitrogen fertilization (Soares *et al*., 2016). While the many species of Rhizobium have been studied in detail for their plant association, another promising bacterium to be studied for its plant association and possible use as biological fertilization are the bacteria from the genus *Bacillus.* It has for instance been reported that *B. subtilis* and *B. mycoides* comprise a major part of the bacterial population in the rhizosphere of established tea bushes. These bacterial colonies could even be found in abundance during non-favorable conditions due to their ability to form spores (Yi, 2018). While these species of *Bacillus* are well known as a soil-borne microorganism, some are either fully or partially endophytic in many plant species. Endophytic species of bacteria are bacteria that can colonize the internal tissues of a plant without causing symptoms. This also means that endophytic species of bacteria, under normal circumstances, do not have any harmful effect on its host (Yi, 2018). Most endophytic bacteria establish a mutualistic symbiosis with its host plant which provides the bacteria with a uniform, nutrient rich niche without competition. In turn the endophytic bacteria will provide the plant with beneficial attributes. One of these beneficial attributes is the production of plant growth promoting compounds, while another is the capability to fix nitrogen from the atmosphere and thus provide the plant with a nitrogen source (Rosenblueth & Martínez-Romero, 2008). Some endophytic bacterial species even help their host plant to deal with harsh biotic and

abiotic stresses like high or low temperature and salt stresses (Card *et al*., 2016). As mentioned, the genus *Bacillus* is found in various plant species due to its broad endo-colonization ability. Some studies have already found *Bacillus* species to play a significant role in the nitrogen fixation needs of some plant species or help with mitigating the effects of stresses. An example is one *Bacillus* species that has been shown to help rice by fixating nitrogen from the air (Sengupta *et al*., 2017). Although *B. mycoides* is a species with the capability of forming endophytic relationships with a variety of plant species, they seem to be unable to endo-colonize species from the *Legume* family. In this thesis we will explore this exclusion of *B. mycoides* from the *Legume* family and test the hypothesis that under stress conditions the inability to infect members of the *Legume* family might be less stringent. At the end of this thesis, we would like to answer the following research question: "Does inoculation with *B. mycoides* affect plant growth (positively or negatively) under stress conditions?"

Materials and Methods

Bacterial culture

In this study *Bacillus mycoides* strain M2E15 was used. This bacterial strain has been isolated from the endosphere of potato (*Solanum tuberosum* cv. Wijster*)* (Yi *et al*., 2016). *Bacillus mycoides* is a rod shaped and chain forming bacterium, associated with the *Bacillus cereus* group which is another endo-colonizing bacterium linked with growth promotion in plants like soybean, wheat and Chinese cabbage (Yi *et al*., 2016; Ku *et al*., 2018).

The *Bacillus mycoides* M2E15 strain has been genetically altered to be resistant to the antibiotic kanamycin (Yi, 2018) enabling the selection on growth media of *Bacillus mycoides* M2E15, specifically.

Stocks of the *B. mycoides* strains used were stored at -20 °C. The nutrient broth used to grow the *B. mycoides* M2E15 for inoculation was made using 5 grams peptone, 3 grams yeast extract and 5 grams sodium chloride added to 1000 ml distilled water. After this its pH was adjusted to 7 (Aryal, 2015). The *B. mycoides* stock was added and grown overnight at 28 °C and 180 revolutions a minute. For use in inoculation the bacteria were suspended in a phosphate buffered saline solution (PBS) which was adjusted to 0.6 at 600nm (PBS, 2006).

For our nitrogen stress and nodulation experiments we also inoculated some plants with rhizobium. To grow the rhizobium, we used a YEM nutrient medium to grow the bacterial culture from a plate. The YEM medium was prepared using 1 gram of yeast extract, 10 grams of mannitol, 0.5 grams of K_2HPO_4, 0.2 grams of $MgSO_4$, 0.1 grams of NaCl and 1 gram of $CaCO_3$ which was added to distilled water until the total volume was raised to be 1000 ml (Yamal *et al*., 2013). After autoclaving the YEM nutrient broth at 120 °C the *Rhizobium* culture was added and grown for 3 days. For use in the inoculation the bacteria were suspended in phosphate-buffered saline solution (PBS, 2006).

For the nodulation experiment five other strains of *Bacillus mycoides* were used. These strains were *B. mycoides* S3E15, *B. mycoides* S2E19, *B. mycoides* SB8, *B. mycoides* SB4 and *B. mycoides* SB13.4 respectively. These bacterial cultures were grown and prepared the same way as *B. mycoides* M2E15 for inoculation of the Green Pea plants used in the nodulation experiment.

Seed germination

For germination of the seeds, they were inserted in sterilized vermiculite in clay pots . The vermiculite was sterilized by putting it into autoclavable bags and autoclaving it for 4 hours at 120 °C. About 90% of seeds started germinating after 5 to 8 days. The clay pots were placed in black plastic containers filled with a layer of demi water when needed. This meant that the seeds were always hydrated during their germination (see fig. 1). Both the clay pots and the containers were sterilized using ethanol before use. The germination of the seeds was achieved in a sterilized climate chamber that was set at a temperature of 21 °C (see fig 1).

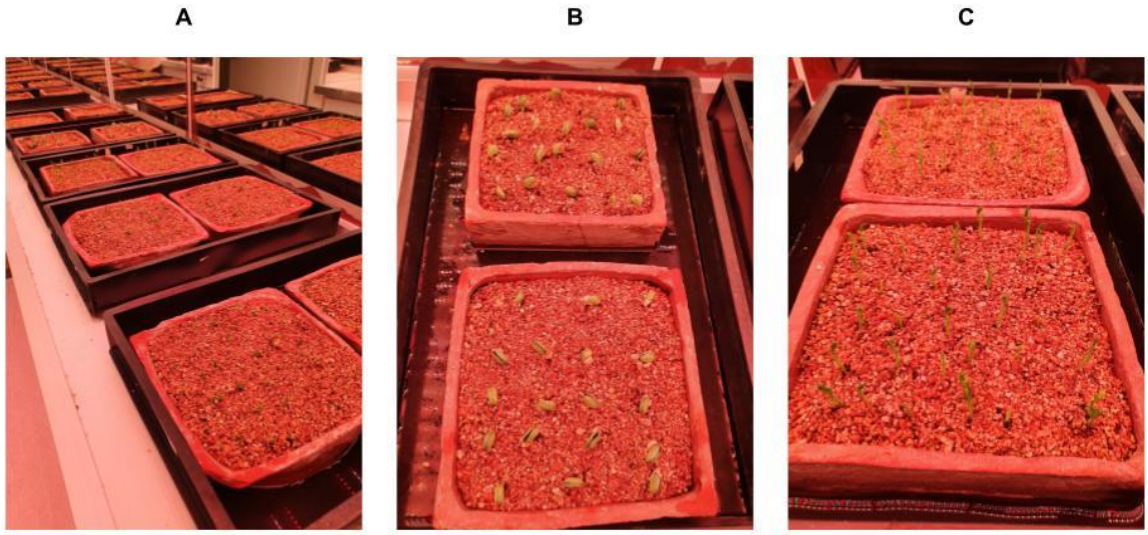

Fig 1: G**ermination setup.** (A) 4 germination boxes were set up next to one another in a cleaned and sterilized climate chamber set at 14 hours of light and at a temperature of 21 ˚C. (B) Germinated broad bean seeds after approximately 5 days. (C) Brown pea germination after approximately 6 days.

During our experiments we used different seeds from nodulating *Legumes*, non-nodulating *Legumes* and non-*Legumes* as control (see fig. 2).

Common name	**Latin name**	**Supplied by**
Green pea	*Pisum sativum*	Vreeken zadenhandel
Brown pea	*Pisum sativum* var. *arvense*	Vreeken zadenhandel
Dwarf bean	*Phaseolus vulgaris*	Vreeken zadenhandel
Broad bean	*Vicia faba*	Vreeken zadenhandel
Weeping boer-bean	*Schotia brachypetala*	Unknown
Silver wattle	*Acacia dealbata*	Vreeken zadenhandel
Sea buckthorn	*Hippophae rhamnoides*	Vreeken zadenhandel
Gray alder (Wild seeds)	*Alnus incana*	Wild seeds
Chinese cabbage	*Brassica pekinensis*	Vreeken zadenhandel
Soya bean	*Soya obelix*	Vreeken zadenhandel
Black locust	*Robinia pseudoacacia*	Vreeken zadenhandel

Fig. 2: **This table shows the species used in our experiments.** All of these were used in our experiments with green pea, Brown pea and Chinese cabbage used as the major seeds in our experiments. Most seeds were bought from private vendors apart from the Alder seeds which are wild seeds taken from wild Alder trees in Groningen.

Endo-colonization

To explore the exclusion of *B. mycoides* M2E15 from legume plants we grew a variety of seeds (see Fig 2.) into seedlings using our germination setup (see Fig 1.). When 90% of planted seeds were germinated (which could take from 5 till 7 days depending on the species) the seedlings were placed into pots that were cleaned using regular dish soap first after which we sterilized the boxes holding the pots using a solution of 70 % ethanol. The pots were filled with 75% potting soil which was unsterilized 12,5% vermiculite and 12,5% unsterilized sand. Vermiculite was added to improve water holding capacity of the mixture, while the sand made it easier to harvest the plants without damaging the roots.

The seedlings were transplanted from the germination setup to the pots. For the uninoculated plants the plants were directly transplanted from the germination set up to the pots

and put into a climate chamber. For the inoculation treatment, the root and stem of the seedlings were first dipped into the PBS solution containing the bacterial cultures before being transplanted into the pots. As we were also interested in the survival rate of *B. mycoides* M2E15 in the soil 250 µL of the PBS solution containing the *B. mycoides* culture was pipetted into the soil at the base of the seedling. Both treatments were placed in different climate chambers to minimize cross contamination.

To have enough replicates in case some of the seedlings would not survive the transplanting each species/treatment would comprise 15 replicates. This means our final endo-colonization experiment consisted of 30 plants of each species/treatment and thus a total of 330 plants. However, due to the limited number of Weeping boer-bean plants (only 8 seeds were available) the total plant count was 308 plants. After 15 days the plants were harvested to be used in our endo-colonization experiments (see Fig. 3).

Fig. 3: **Climate chamber after harvesting.** After the harvesting only the extra brown peas (Right) and green peas (Left) were left in the climate chamber. As can be clearly seen these were already quite large on day 15.

For the visualization of the rate of endo-colonization samples of the plant and soil were plated onto agar containing kanamycin. The agar used was made using the same composition as the nutrient broth used for the inoculation. Five grams peptone, 3 grams yeast extract, 5 grams sodium chloride and 15 grams of Agar was added to 1000 ml of distilled water. This solution was then autoclaved at 120 °C and kept in an incubator at 50 °C to keep it from solidifying (Aryal, 2015). Before pouring the plates we also added the kanamycin stock to obtain a final concentration of 50µg/ml. Before plating, the soil particles were suspended in a PBS solution (see bacterial culture) to stabilize the bacteria living in the soil. The plant parts either a piece of root, stem or leaf were cut into small pieces before being sterilized using a bath of 70% ethanol followed by a bath of 3% sodium hypochlorite after which the remaining ethanol and sodium hypochlorite was washed of using sterilized distilled water. The plates were then put outside in the lab at 21 °C for 3 days after which we observed the plates for any growing of *B. mycoides* (see Fig. 4).

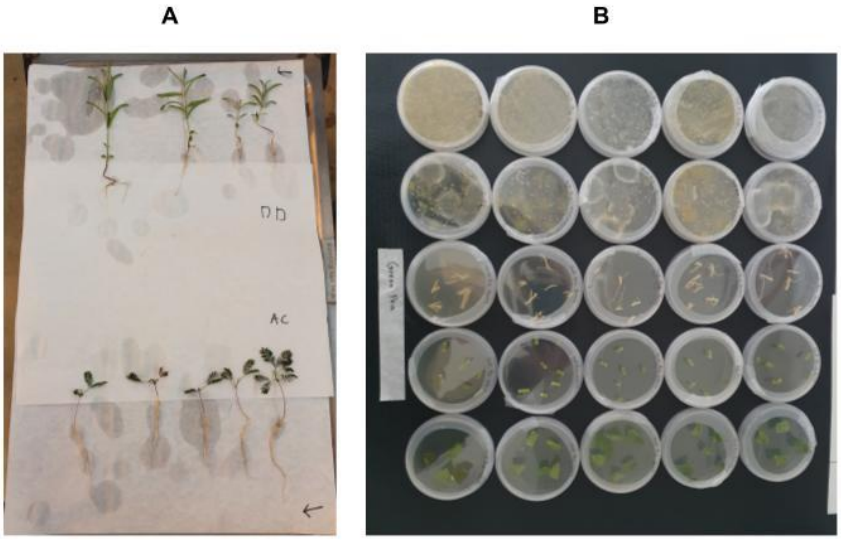

Fig. 4: **Our endo-colonization experiment.** (A) Sea buckthorn (Top) and Silver wattle (Bottom) cleaned and ready to be cut, sterilized and put on a plate. (B) plates using green pea with clear growth of mold in the unsterilized bulk soil (Top) and rhizospheric soil (One down from top). However, from top to bottom, the roots, shoots and leaves do not show any growth.

To measure growth, the plants were harvested and cleaned (see Fig. 5). The root was separated from the rest of the plant and weighed. The shoot length was measured, and the plant matter was weighed. To get a dry weight measurement we put the plant matter into a stove at 70 °C overnight before weighing the roots and stems separately. For the nodule weight measurements, the nodules were separated from the root material and weighed. After an initial fresh weight measurement, they were dried in the stove and a dry weight measurement was taken. These measurements were analyzed by using IBM's SPSS from New York for statistical analysis and graphed using GraphPad Software Inc. Prism from New York.

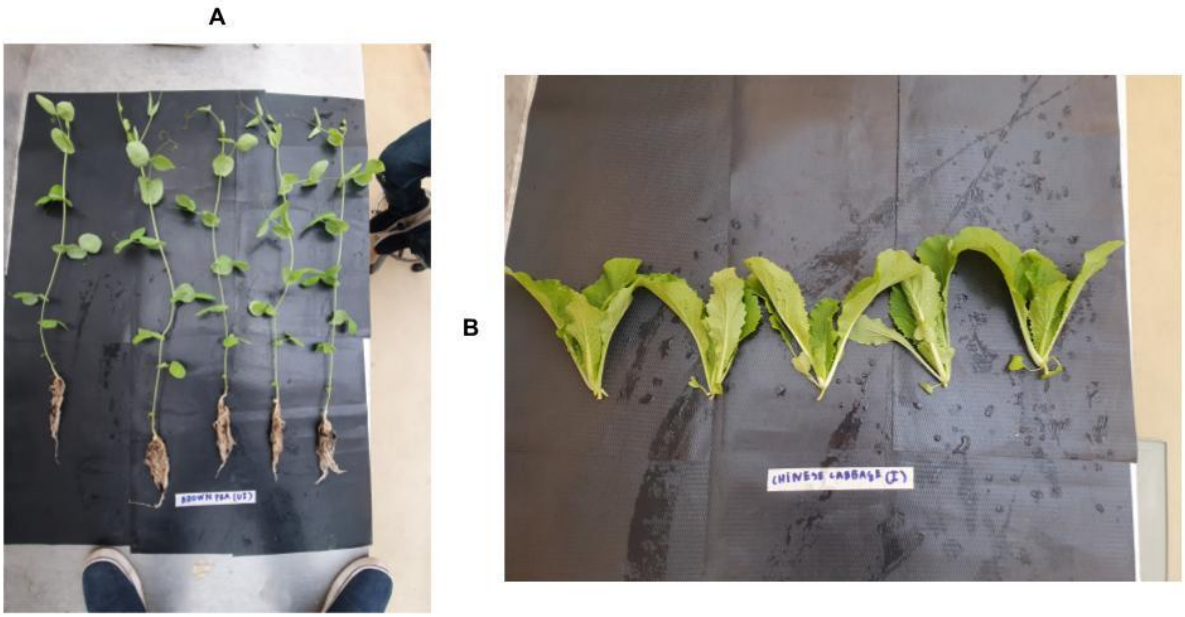

Fig 5: **Plants after cleaning for the plant parameter measurements.** (A) Brown Pea plants after cleaning, 5 replicates used. (B) Chinese cabbage plants after cleaning. Due to the nature of the roots of Chinese cabbage the roots were damaged and discarded and thus only the shoot length and weight was used to compare the two treatments.

Salinity stress

To explore if *B. mycoides* M2E15 can endo-colonize plants from the legume family under stress conditions, the plants were grown under conditions of stress. To grow the seedlings used in this experiment we used the same method as during the endo-colonization experiment. After 5 to 7 days, we transplanted the seedlings into soil made from 70% potting soil, 12,5% vermiculite and 12,5% sand. As the seedlings would undergo salinity stress conditions the three salt treatments were made up of a treatment with 0 mM NaCl, 50 mM NaCl and 100mM NaCl. The plants were given 30 ml of these solutions every day per plant to induce osmotic stress to the plants. We also had two treatments concerning the inoculation with *B. mycoides* M2E15. Due to this we were able to compare the plant parameters with and without inoculation of *B. mycoides* M2E15 in the soil. As we found a possible inhibiting effect of *B. mycoides* inoculation on the growth of nodules, we also inoculated the plants with *Rhizobia* to make sure all plants received an inoculation and not to be dependent solely on wild inoculation from the soil or air used. After 15 days (about 2 weeks) the plants were harvested and used to check for endo-colonization using the same method as described above (see Fig. 6).

Fig. 6: **Salinity stress green pea plants after 15 days of growing.** (A) Green pea plants that are uninoculated after 15 days (about 2 weeks) of growing. (B) Green pea plants that are inoculated after 15 days (about 2 weeks) of growing.

The plant parameters, nodule weight measurement and endo-colonization data were obtained using the same methods used in our endo-colonization experiments (see fig. 7). To help visualize the effect of *B. mycoides* M2E15 on nodulation and causally connected plant growth and organic matter production, we also looked at the total chlorophyll content in the leaves of the green pea plants. Chlorophyll content has been directly linked to plant growth and production and the lack of nodules in a legume plant could inhibit growth due to a lack of nitrogen (Opabode & Akinyemiju, 2007). The Chlorophyll content of our green pea plants was measured by suspending 25 mg of leaf material in 10 ml of DMSO overnight. The resulting solution was then measured using a spectrophotometer at 663, 645 and 470 nm (Barnes *et al*., 1992). These measurements were analyzed by using IBM's SPSS from New York for statistical analysis and graphed using GraphPad Software Inc. Prism from New York.

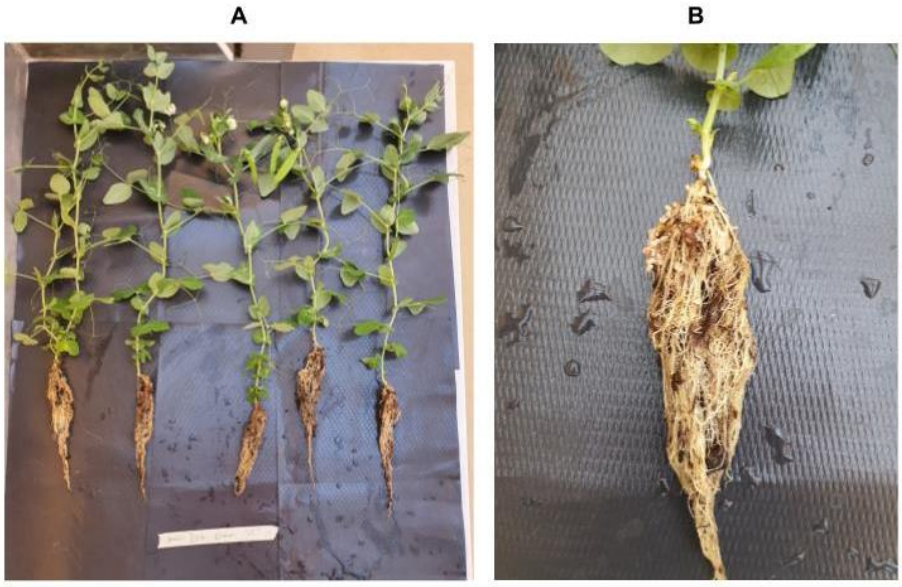

Fig. 7: **salinity stress green pea harvested after 15 days for plant parameter measurement and nodule measurement.** (A) Green Pea uninoculated after 15 days of 0mM salinity stress treatment. (B) Green pea root showing clear signs of active nodulation.

Nitrogen stress

Another stress we tested for its effect on endo-colonization was nitrogen limitation. For this experiment the green pea seeds were germinated using the same method we used during our initial endo-colonization experiment. As we were interested in the effect of *B. mycoides* M2E15 on the growth of the green pea plants we used two different climate chambers for the two different, inoculated with *B. mycoides* M2E15 and uninoculated with *B. mycoides* M2E15, treatments. To have control on the amount of nitrogen administered to the plants we transferred the plants into pots containing only sterile vermiculite after the germination was completed so there would be no nitrogen in the soil. For each of the two *B. mycoides* treatments 4 nitrogen treatments were used (see Fig. 8). These treatments consist of a nitrogen component and a *Rhizobium* component where either a nutrient solution with or without nitrogen was given and the plant was inoculated or not inoculated with *Rhizobium*. To minimize the cross contamination with *Rhizobium* The inoculated plants were separated in the climate chamber from the uninoculated plants.

Nitrogen Treatment	**Replicates *(times 2 for both inoculated and uninoculated)***	**Location in Inoculated climate chamber**	**Location in uninoculated climate chamber**
Nitrogen - & *Rhizobium* -	15	Front & Right side	Front & Right side
Nitrogen + & *Rhizobium* -	15	Front & Right side	Front & Right side
Nitrogen - & *Rhizobium* +	15	Back & Left side	Back & Left side
Nitrogen + & *Rhizobium* +	15	Back & Left side	Back & Left side

Fig 8: **The 4 different nitrogen treatments.** These nitrogen treatments were made using nutrient solution containing nitrogen where nitrogen is **+** and without nitrogen where nitrogen is **-**. The *Rhizobium* treatment was made using the same inoculation step used for *B. mycoides* M2E15. To minimize cross contamination the *Rhizobium* treated plants were kept away from the *Rhizobium* – treatment.

After 15 days the plants were harvested for the visualization of the endo-colonization (see Fig. 9). This was visualized using the same method used during our endo-colonization experiment. After the endo-colonization visualization experiment we again took the measurements of the plant parameters, nodulation and chlorophyll content using the same methods used during the endo-

colonization and salt stress experiments. These measurements were analyzed by using IBM's SPSS from New York for statistical analysis and graphed using GraphPad Software Inc. Prism from New York.

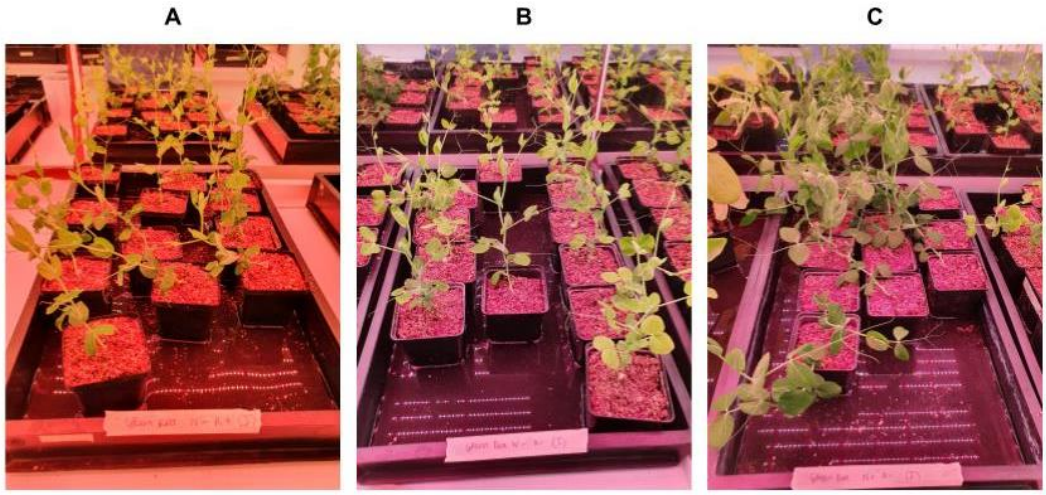

Fig. 9: **Green pea plants after 15 days of being subjected to nitrogen stress.** (A) Nitrogen – and *Rhizobium* + treatment after 15 days. (B) Nitrogen – and *Rhizobium* – treatment after 15 days. (C) Nitrogen + and *Rhizobium* – treatment after 15 days.

Nodulation

Due to the preliminary observations made, we were very interested in the possibility of *B. mycoides* inhibiting the formation of nodules. We designed an experiment using different strains of *B. mycoides*, besides M2E15: SB4 and SB13.4 (strictly soil based); S2E19 and S3E15 (endo-colonization) and SB8 (intermediate) (Yi, 2018). To check if the effect came from the *B. mycoides* in the soil or that the effect on nodulation was coming from *B. mycoides* trying to endo-colonize the different strains were used to check the effect of inoculation on nodulation in green pea. The setup used was the same as the setup used in the nitrogen stress experiment (see Fig. 10).

Treatment	**Replicate plant number**
Inoculating with *Rhizobium* but uninoculated with *B. mycoides* M2E15*(T1)*	20
uninoculated with both *Rhizobium* and *B. mycoides* M2E15 *(T2)*	20
Inoculating with *B. mycoides* S3E15 and *Rhizobium (T3)*	15
Inoculating with *B. mycoides* S2E19 and *Rhizobium (T4)*	15
Inoculating with *B. mycoides* SB8 and *Rhizobium (T5)*	15
Inoculating with *B. mycoides* SB4 and *Rhizobium (T6)*	15
Inoculating with *B. mycoides* SB13.4 and *Rhizobium (T7)*	15

Fig. 10: **The 7 treatments used in our nodulation experiment.** In this experiment we are using 5 different strains of *B. mycoides* to check for the existence of an inhibiting factor in nodulation formation in green pea.

Again, the seeds of the green pea were germinated using the same method used in our endo-colonization experiment. After 5 to 7 days the seeds were transplanted into a soil mix of 75% potting soil, 12,5% vermiculite and 12,5% sand. After growing for 15 days the plants were harvested (see Fig. 11) and measurements on the plant parameters, nodule weight and chlorophyll content were done using the same method used in the nitrogen stress experiment. These measurements were analyzed by using IBM's SPSS from New York for statistical analysis and graphed using GraphPad Software Inc. Prism from New York.

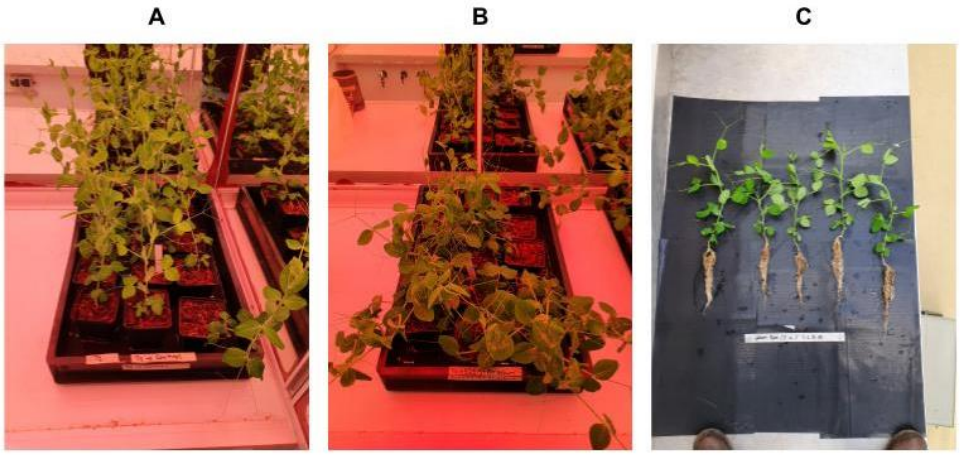

Fig. 11: **Nodulation experiment after 15 days (about 2 weeks) of growing.** (A) Control 1 after 15 days of growing. (B) Green pea with *B. mycoides* S2E19 treatment after 15 days of growing. (C) Green pea plants harvested after 15 days of growing after being inoculated with *B. mycoides* S2E19.

GFP tagging.

To help with the visualization of the exclusion of *B. mycoides* M2E15 in *Legumes* we also explored the use of GFP tagging. This was done by first germinating green pea and Chinese cabbage seeds using the same method used in the other experiments. After the germination step, we transplanted 10 Green pea and 10 Chinese cabbage seedlings into pots filled with sterile vermiculite. Before transplanting the seedlings, we again inoculated them with *B. mycoides* M2E15 for 30 minutes. The *B. mycoides* M2E15 used in this experiment was genetically altered to display Green Fluorescent proteins and could thus be used to visualize them inside the tissue of plants with GFP confocal microscopy (Yi, 2018).

After 15 days the plants were harvested for their roots. These roots were sterilized using the same method used during our endo-colonization experiments. This meant we first cleaned them using demi water, then sterilized them using 70% ethanol and 3% Sodium Hypochlorite baths for 2 minutes each. After these treatments we cleaned the Sodium hypochlorite of the root hairs using sterile distilled water and made the microscope slides suspending the small root hairs in sterile distilled water. The fluorescence images were captured with a confocal microscope (LSM800; Carl Zeiss, 2022) equipped with a 32- channel gallium arsenide phosphide photomultiplier tube (GaAsP-PMT), Zen 2009 software (Zeiss, 2022), and a 63 × 1.40 NA objective (Zeiss, 2022). The pictures were analyzed broadly by using ImageJ.

Results

Endo-colonization

Our endo-colonization experiments had two parts to it. The first part was aimed at visualizing *B. mycoides* M2E15 inability to endo-colonize plants from the *Legume* family. This showed us that the preliminary research done on this phenomenon was indeed correct (Yi, 2018). We did find *B. mycoides* M2E15 growing on plates harboring bulk or soil from the rhizosphere, showing that our *B. mycoides* strain could survive in the soil. Sea buckthorn was the only member of the Legume family that showed a positive sample. Our control, Chinese cabbage, did show clear endo-colonization by *B. mycoides* through all 5 replicates (see Fig. 12).

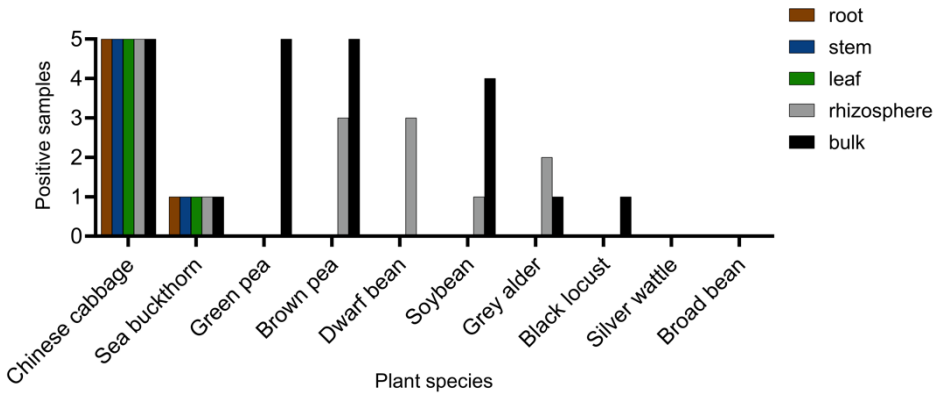

Fig. 12: **Recovery of vital Bacillus mycoides M2E15 from soil and plant organs for cultures of different legume and non-legume plant species.** As can clearly be seen the plants from the *Legume* family were not endo-colonized by *B. mycoides* M2E15 while our control using Chinese cabbage did show clear growth of *B. mycoides* M2E15 on the plates.

When looking more in depth into three of the above-mentioned species of plants we did not find any significant difference between plants that were inoculated with *B. mycoides* and plants of the same species that were uninoculated with *B. mycoides*. All plant parameters (see Fig 13. & appendix 1.1) that were measured were found to be not significantly different in both instances. Even Chinese Cabbage which was endo-colonized by *B.* mycoides (see Fig. 12) did not show a significant increase in size or mass when comparing it to the control. While Brown pea did show a difference in shoot fresh weight at first glance this difference was not found to be significant (see Fig. 13 & appendix 1.1).

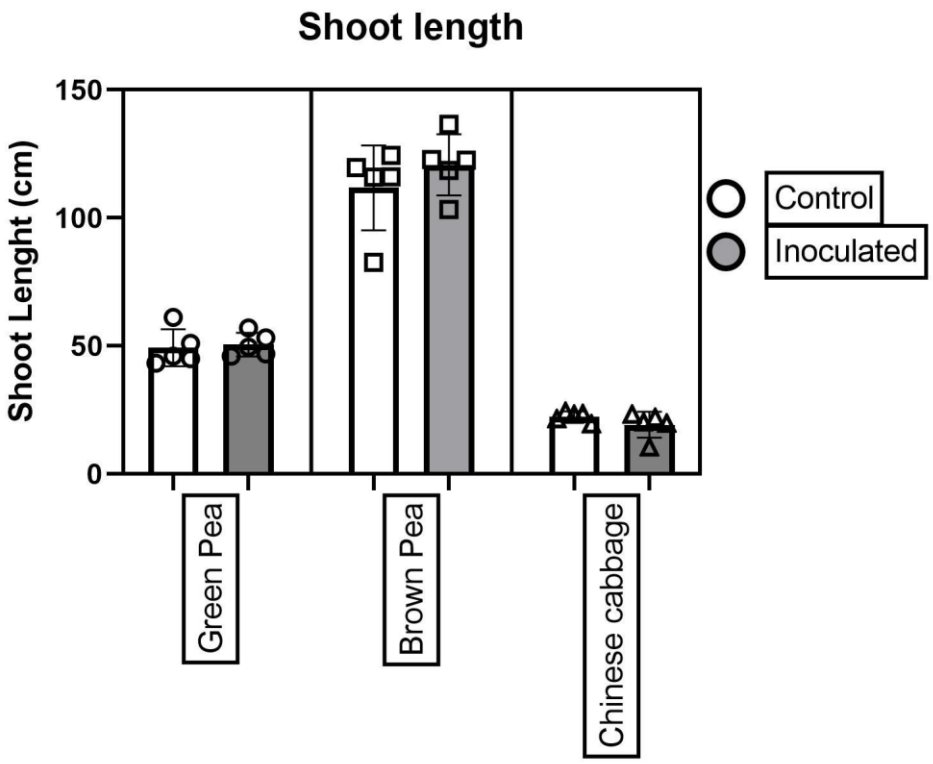

Fig 13: **Shoot length parameters From different legume and non-legume plant species and different inoculation treatments.** There was no significant difference found between the inoculated and control treatment.

While the plant parameters like size and weight did not show any significant difference between the control and inoculated treatments, we did find a difference when looking at the effect *B. mycoides* inoculation had on the formation of nodules on the root of the green and brown pea plants. This significant difference shows a possible inhibition in the ability to form a relationship between *Rhizobia* and the green and brown pea plants (see Fig. 14).

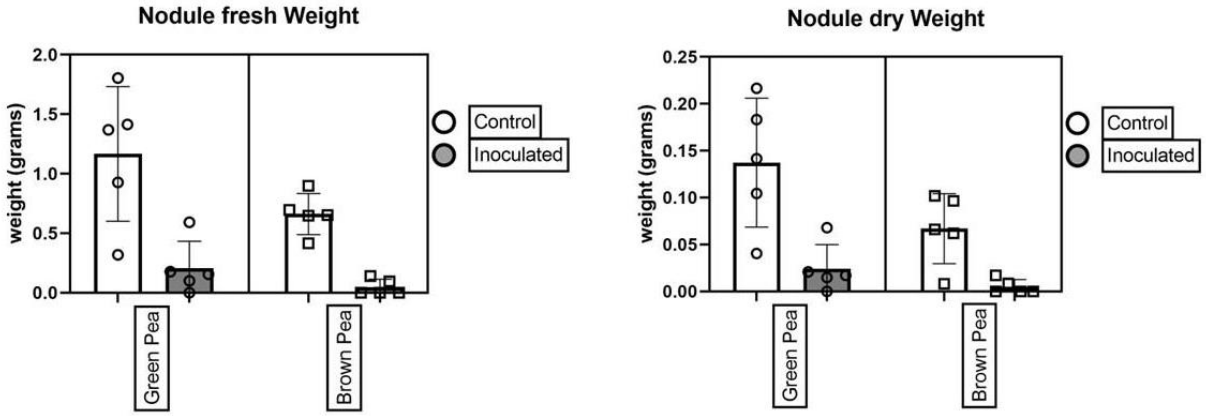

Fig. 14: **Nodulation data from two legume plant species and different inoculation treatments.** We found a significant difference between the two *B. mycoides* M2E15 treatments.

Salinity stress

By using salinity stress we hoped to overcome the defenses against endo-colonization by *B. mycoides* M2E15 in legume plants. However, no endo-colonization was seen in green pea after 15 days. There

was a difference seen between our initial endo-colonization experiment and the salinity stress experiment as there is a lack of *B. mycoides* in the rhizospheric soil and bulk soil (see appendix 1.2).

When analyzing the difference between the plants from the uninoculated and inoculated treatments we found that there was no significant difference between the two *B. mycoides* treatments. The treatment with *B. mycoides* had no effect on the growth or ability of green pea to cope with the salinity stress (see Fig. 15 & appendix 1.3). The significant difference in nodulation seen during the endo-colonization experiment was not observed during our salinity stress experiments in the 0mM and 50 mM treatments while the 100 mM salinity treatment did show a significant difference in nodulation where the uninoculated plants showed a higher number of nodules by weight (see Fig. 16).

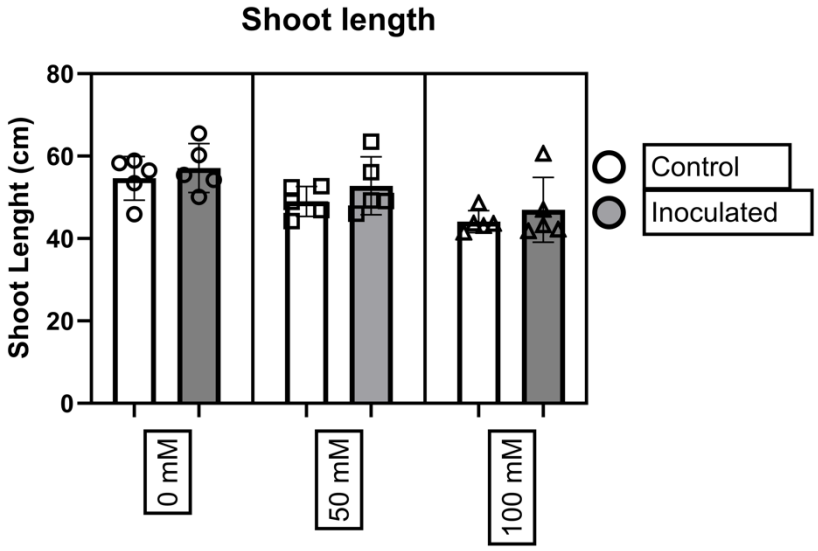

Fig. 15: **Shoot length parameter from different salt stress variables and different inoculation treatments.** The graph shows a clear difference between the plant parameter shoot length under different salinity conditions while not showing a difference between *B. mycoides* treatments.

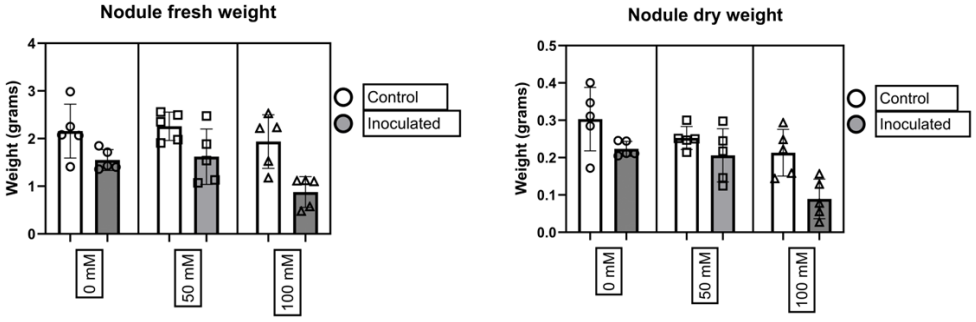

Fig. 16: **Nodulation data from different salt stress variables and different inoculation treatments.** We found no significant difference between the two *B. mycoides* M2E15 treatments in the 0 mM and the 50 mM treatments. The 100 mM treatment did show a significant difference in nodulation by weight.

This effect on nodulation in the 100 mM salinity treatment did not affect the chlorophyll content of the plant, however. We did not find any significant difference in chlorophyll content between the *B. mycoides* treatments while a significant difference between salinity treatments was seen (see Fig. 17).

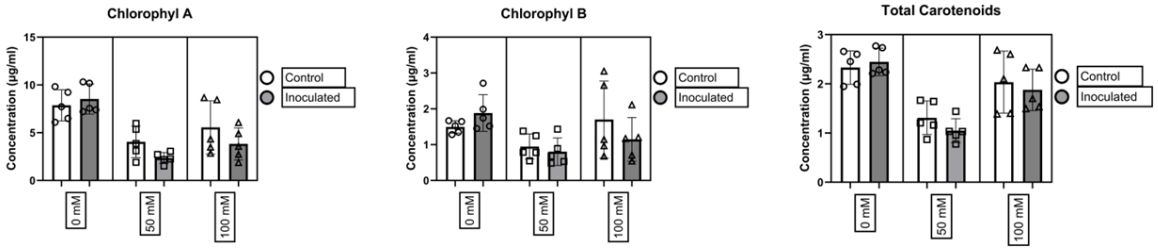

Fig. 17: **Chlorophyll data from different salt stress variables and different inoculation treatments.** We found no significant difference between the two *B. mycoides* M2E15 treatments.

Nitrogen stress

As *B. mycoides* M2E15 is known to be able to help plants by fixing nitrogen from the air, we tried to use nitrogen stress to overcome the exclusion of *B. mycoides* M2E15 in green pea plants (Yi, 2018). When looking at the infection data *B. mycoides* M2E15 is still excluded from the green pea plants used. There was also no growth seen in the bulk soil and soil from the rhizosphere (see Appendix 1.4).

The difference between the uninoculated and inoculated plants was analyzed to check if the treatment using *B. mycoides* M2E15 was influencing the growth of the plants. The plant parameters show no significant differences between the uninoculated and inoculated treatments (see Fig. 18 & Appendix 1.5). The nodule fresh weight (p = 0,046) and nodule dry weight (p = 0,034) do show a significant difference between the inoculated and uninoculated treatments with the uninoculated treatments showing a higher weight of total nodules (see fig. 19).

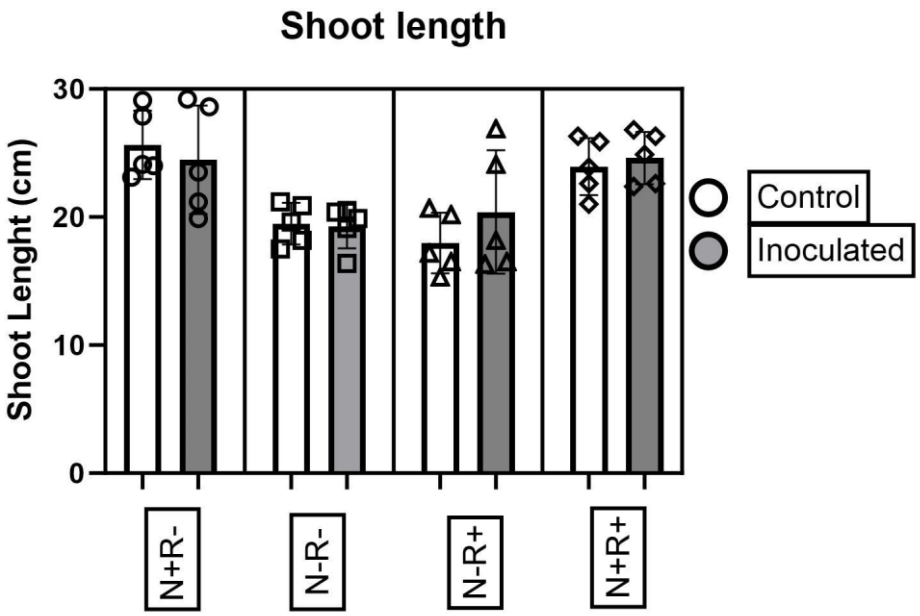

Fig. 18: **Shoot length parameter from different nitrogen stress variables and different inoculation treatments.** The graph shows a clear difference between the plant parameter shoot length under different nitrogen conditions while not showing a difference between *B. mycoides* treatments.

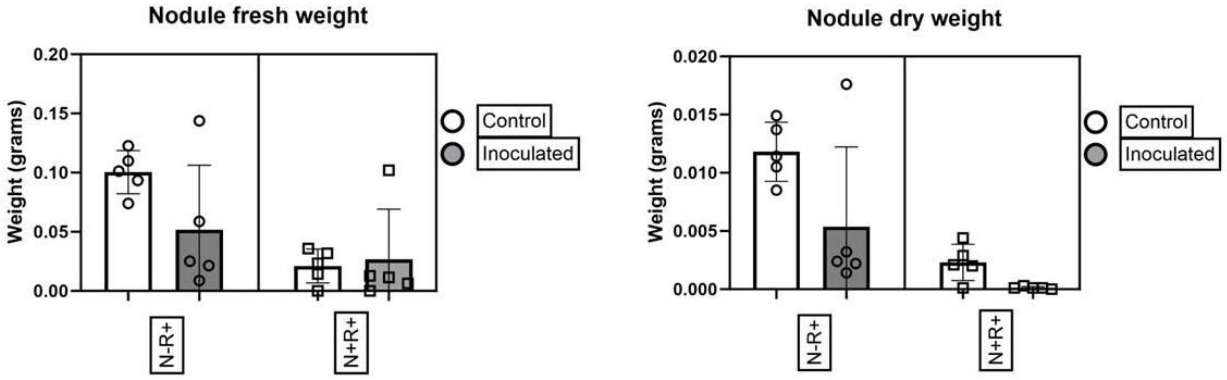

Fig. 19: **Nodulation data from different nitrogen stress variables and different inoculation treatments.** We found a significant difference in nodulation by weight between the two *B. mycoides* treatments.

The results showing a significant difference between the nodulation from the uninoculated and inoculated treatments again show up in our chlorophyll data. Here the different concentrations showed a significant difference between *B. mycoides* treatments (see fig. 20).

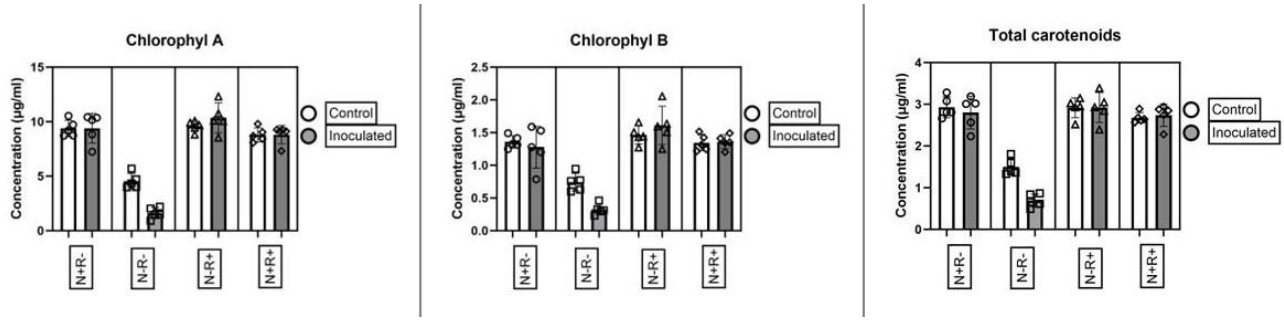

Fig. 20: **Chlorophyll data from different nitrogen stress variables and different inoculation treatments.** We found a small but significant difference between the different *B. mycoides* treatments.

Nodulation

When looking at the effects of *B. mycoides* on the nodulation in green pea we found no significant effect on plant growth (see Appendix 1.6 & 1.7). However, we did find a significant difference when looking at the effect on nodulation ($p < 0{,}001$). When further exploring the data with multiple comparisons we found that this effect was only seen when considering our T1 control. When we did not include the T1 control we found that nodule fresh weight ($p = 0{,}437$) and nodule dry weight ($p = 0{,}312$) showed no significant differences between the treatments (see fig. 21).

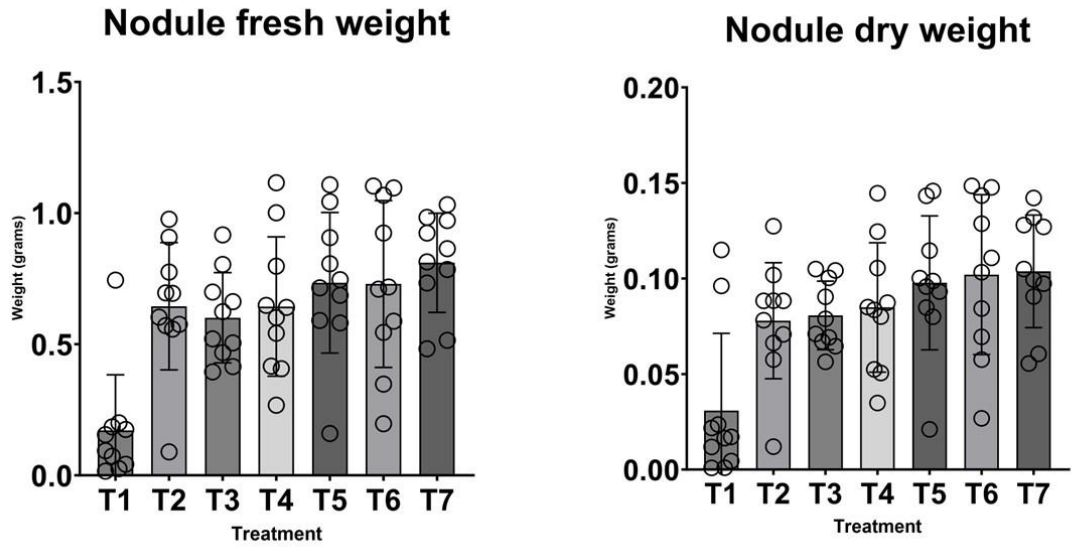

Fig. 21: **Nodulation data using multiple different *B. mycoides strains*.** There was only a significant difference found between all treatments and treatment 1. When taking out treatment 1 we did not find a significant difference between the treatments.

We also investigated the effect on chlorophyll concentrations. And found that there was a significant difference between the treatments and the chlorophyll concentration. When taking out the T1 control we did not find a significant difference between the treatments (see Fig. 22).

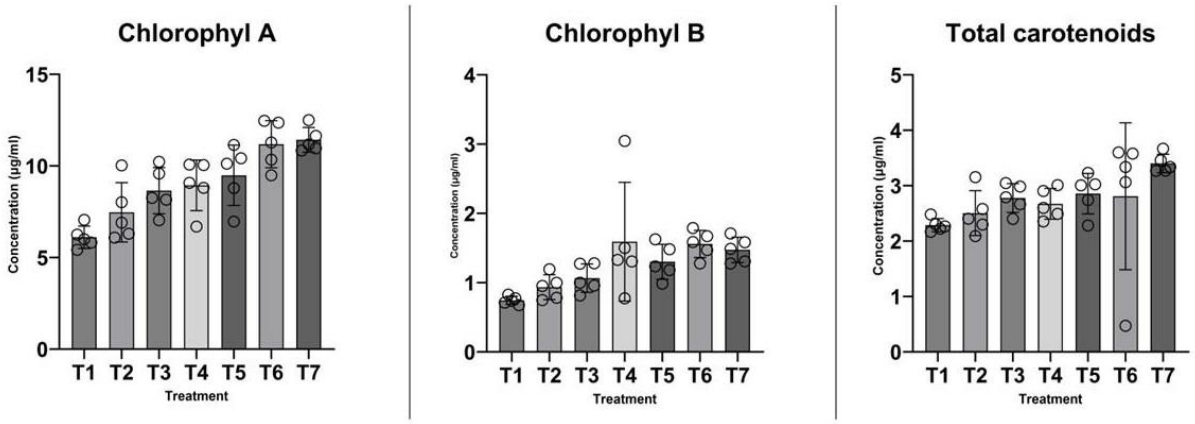

Fig. 22: **Graphs showing the chlorophyll data from our nodulation experiments.** We found a significant difference between the treatments and treatment 1. When taking out treatment 1 we found no significant difference between the different treatments.

GFP tagging.

To help visualize the *B. mycoides* that have endo-colonized the plant cells of Chinese cabbage and possible endo-colonization of green pea plants we used GFP tagging and confocal microscopy to visualize *B. mycoides* M2E15 in both green pea and Chinese cabbage (see fig. 23).

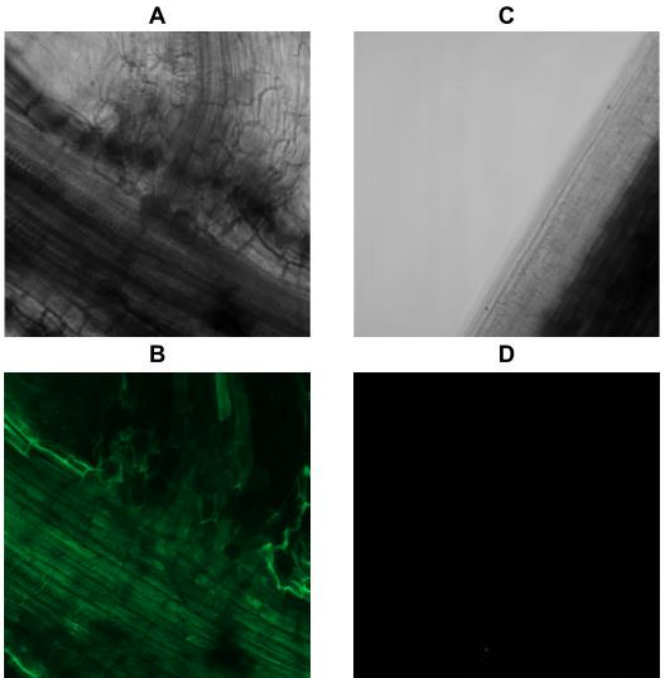

Fig. 23: **GFP confocal scanning pictures.** (A) Picture showing the root of Chinese cabbage. (B) Picture showing the GFP tagged cells of *B. mycoides* M2E15 in the cells seen in picture A. (C) Picture showing the root of a green pea plant. (D) picture showing no GFP tagged cells from *B. mycoides* M2E15 in the cells of the root from picture C.

Conclusion and discussion

Exploring the exclusion of *B. mycoides* M2E15 in members of the *Legume* family of plants showed that *B. mycoides* does not endo-colonize the plants used. While we did observe that the plants were affected by stressors like salt and nitrogen this did not change the relationship between the *Legume* plants and *B. mycoides*, and thus the effect of the stressors were not mitigated, and the stressors still affect plant growth. While no effect on plant growth was found we did observe one possible effect *B. mycoides* inoculation had on the plants from the *Legume* family. We observed an effect on the formation of nodules in the root system of the inoculated plants. However, even in treatments where a significant difference in nodulation by weight was found (100 mM treatment) this difference did not translate into an effect on the other plant parameters or even the photosynthetic capabilities. This means that the effect on nodulation, even when significantly different, did not inhibit the plants' ability to gather sufficient nitrogen. While there was no overall significant effect of *B. mycoides* on the formation of nodules by weight, there was a difference in size and number of nodules. However, this difference in size and number was not measured. While the nodules in the control treatments seemed to be small and spread out throughout the entire root system, the nodules on the inoculated plants were bigger and more concentrated to the spots where inoculation with *Rhizobium* was done. While the weight of these nodules in total was thus comparable and had no significant difference, observing this change in location, size and number does hint to a possible effect on the migration and further colonization of the root structure by nodule forming bacteria like *Rhizobium*. Further exploration into the relationship between *Rhizobium*, *B. mycoides* and the species of *Legume* plants is needed. One possible explanation for the difference is that plants from the *Legume* family see *B. mycoides* not as a beneficial bacterium but as a pathogen and act accordingly (Teixeira *et al*., 2019). Some changes plants go through under stress conditions like pathogen pressure is the production of antioxidants to inhibit the growth of the bacterial colonies that are harmful to the plant. However, these antioxidants are not species specific and will also inhibit growth or outright kill bacterial species that are either not harmful or even beneficial to the plant (Kaur *et al*., 2022). This effect on bacterial growth could explain the lack of nodules further down the root structure as *Rhizobium* that tried to migrate by cell division outside nodules were affected by the

antioxidants secreted by the plant to fight off the *B. mycoides*. The nodules that were present therefore grew to mitigate the effect of having less nodules thus no effect on other plant parameters or photosynthetic capabilities were found (Kaur *et al*., 2022).

Coming back to the effect on plant parameters by *B. mycoides* in Chinese Cabbage is also necessary to put the effect of *B. mycoides* inoculation in perspective. While inoculation with *B. mycoides* does not affect the growth and photosynthetic capabilities of plant from the *Legume* family even when considering the possible effect on nodulation, Chinese Cabbage plants that were endo-colonized also showed no significant effect from this endo-colonization on the growth parameters. When putting this information together *B. mycoides* does not seem to promote growth in any significant capacity. However more in-depth research into stress mitigation by *B. mycoides* is needed to be able to fully conclude if *B. mycoides* does indeed promote growth under different circumstances, or if the endo-colonizing ability is only beneficial to the bacteria and not beneficial to the plant.

When looking at the data the number of replicates and days of growth also need to be taken into account. The replicates were the same for all stress experiment (5 each), however the days that the plants were grown were between 10 and 25 days. While during each experiment the days of growth were consisted this was not the case between different experiments. This means that the experiments cannot be compared to each other, meaning we cannot conclude anything about *B. mycoides* inoculation on a broader scale. The number of replicates also sometimes showed a large spread with outliers that grew slower or faster than the other plants in the experiments. This large spread could be the reason we did not find any significant differences, however more research with larger replicate size is needed to confirm this.

While more research is needed into the exclusion of *B. mycoides* in *Legume* plants, especially on the ground of the disruption of nodule formation, this thesis gives a good starting point for any future research. For further research I would urge a larger sample size and a deviation from nodule weight to counting the nodules and recording their size for a more in-depth look. Another possibility for researching the disruption into nodulation is plating rhizobium into plates made with the root exudate from the control and inoculated plants to see if this disrupts bacterial growth in *Rhizobium* in any way (Kaur *et al*., 2022).

Acknowledgment

First, I would like to thank Professor Theo Elzenga for allowing me to do this research at his department. During my time I have learned a lot and have enjoyed the company and feedback received. Even when my health was in the way Theo immediately responded with kind words and encouragement.
I also would like to thank Henk Jan Venema for helping me with feedback and access to the climate chambers used during our experimentation.
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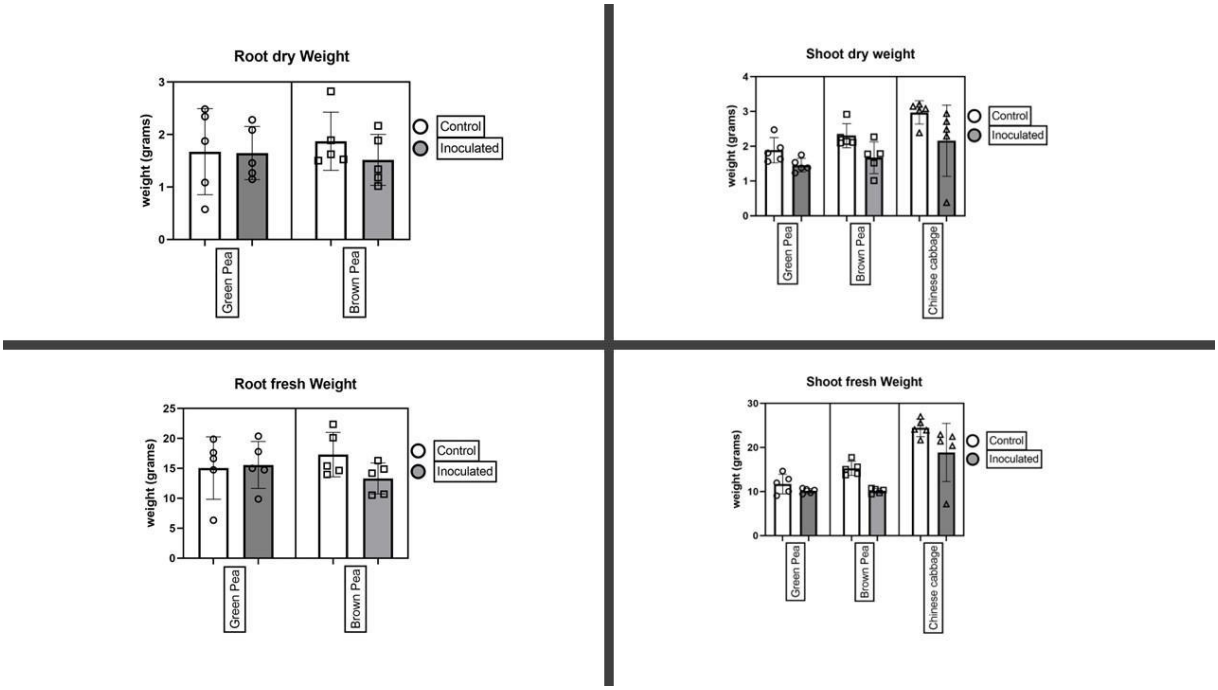
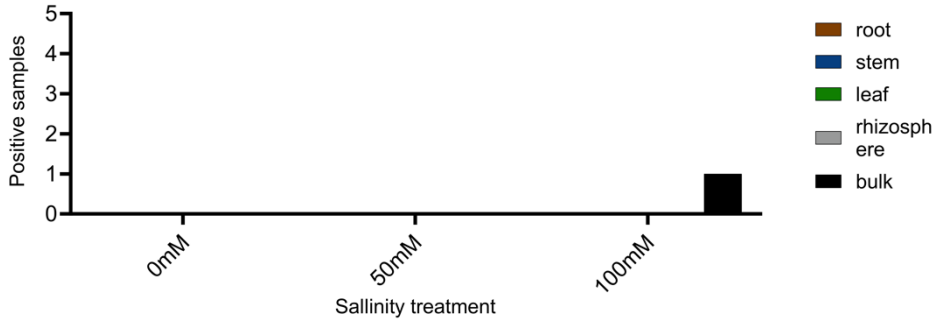
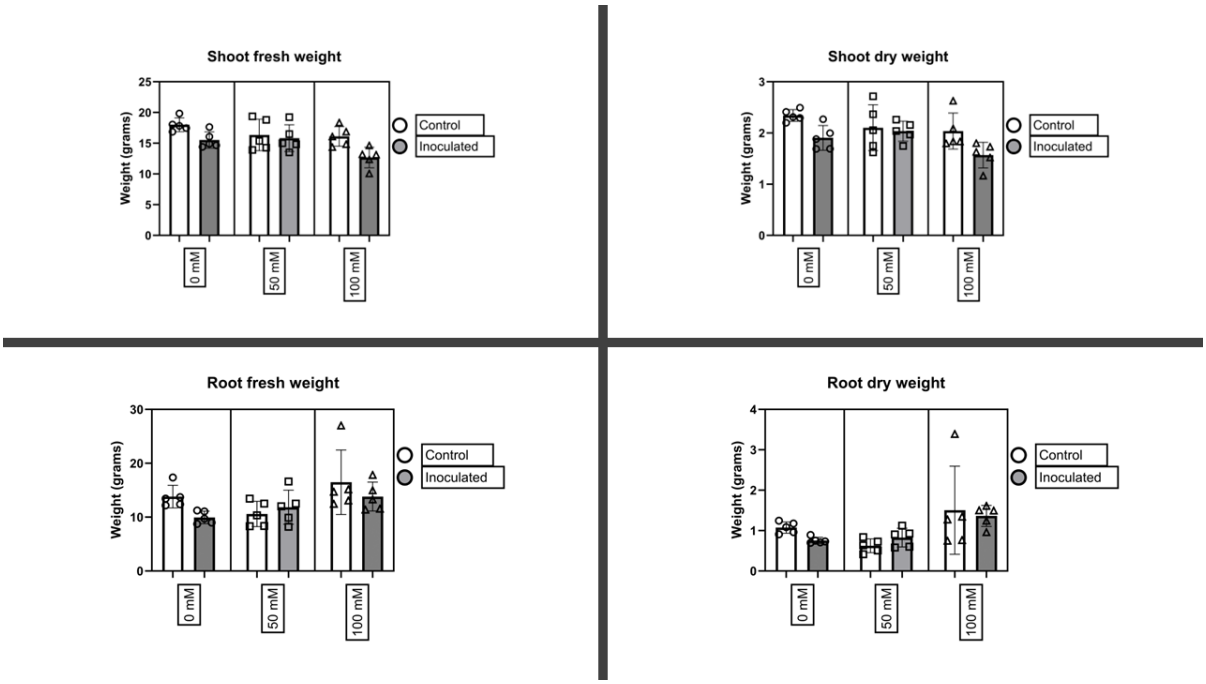
Appendix 1: Plant parameters and infection data



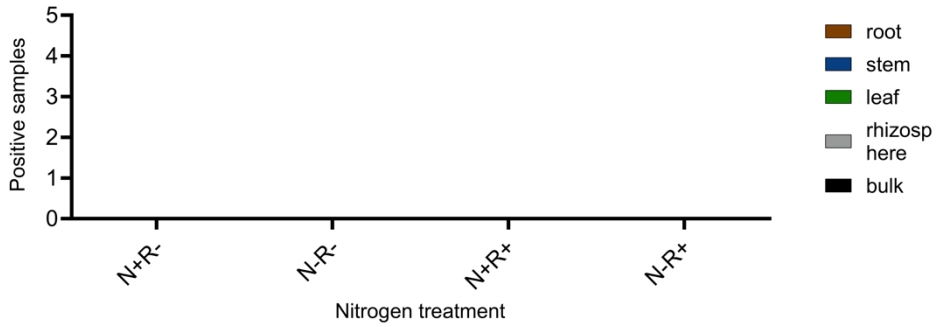
Appendix 1.1: **Graph showing the shoot and root weight parameters of our endo-colonization experiment.** While there seems to be a difference between the inoculated and control treatments this difference is not significant.



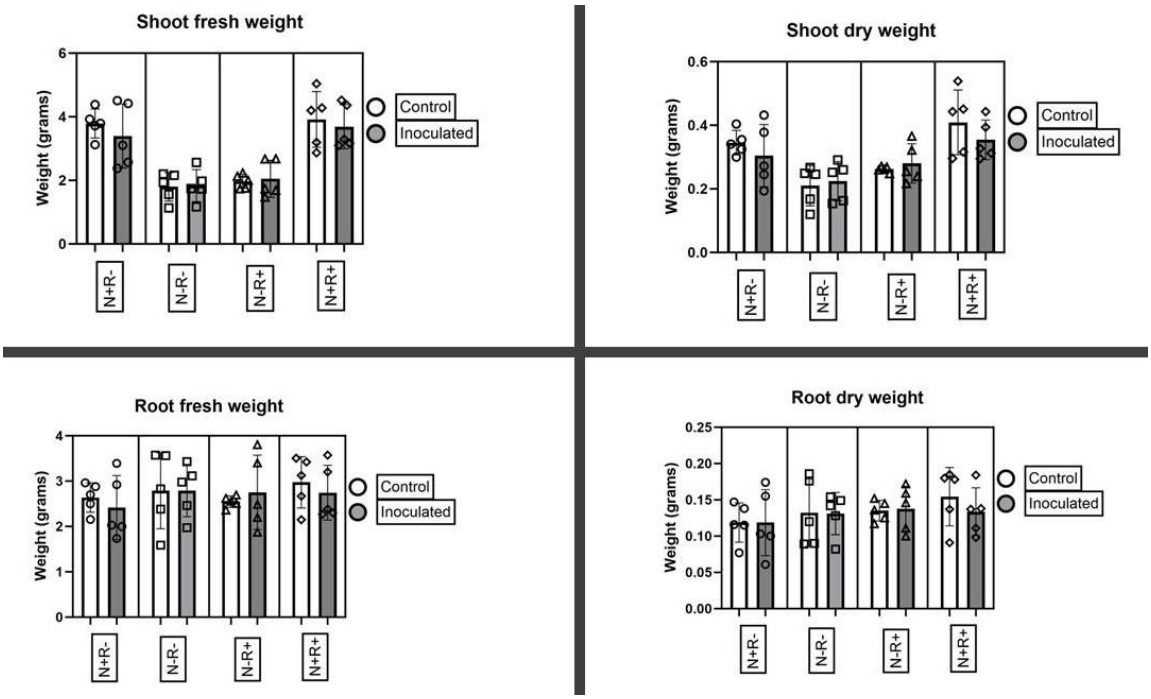
Appendix 1.2: **Table showing the results from our endo-colonization experiment subjecting green pea to salinity stress.** The table shows clearly no growths on almost all plates showing that during salinity stress *B. mycoides* M2E15 is still excluded from endo-colonizing green pea.



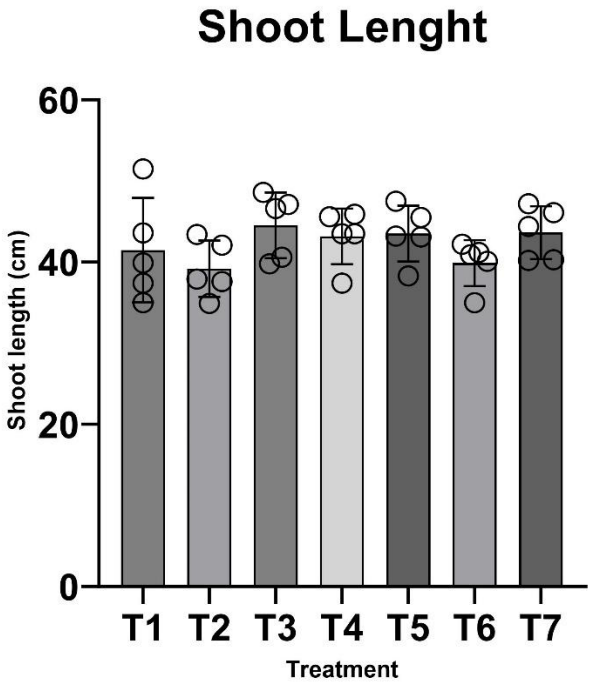
Appendix 1.3: **Graph showing the plant weight parameters from our salinity stress experiment.** The graph shows a clear difference between the plant weight parameters under different salinity conditions while not showing a difference between *B. mycoides* treatments.



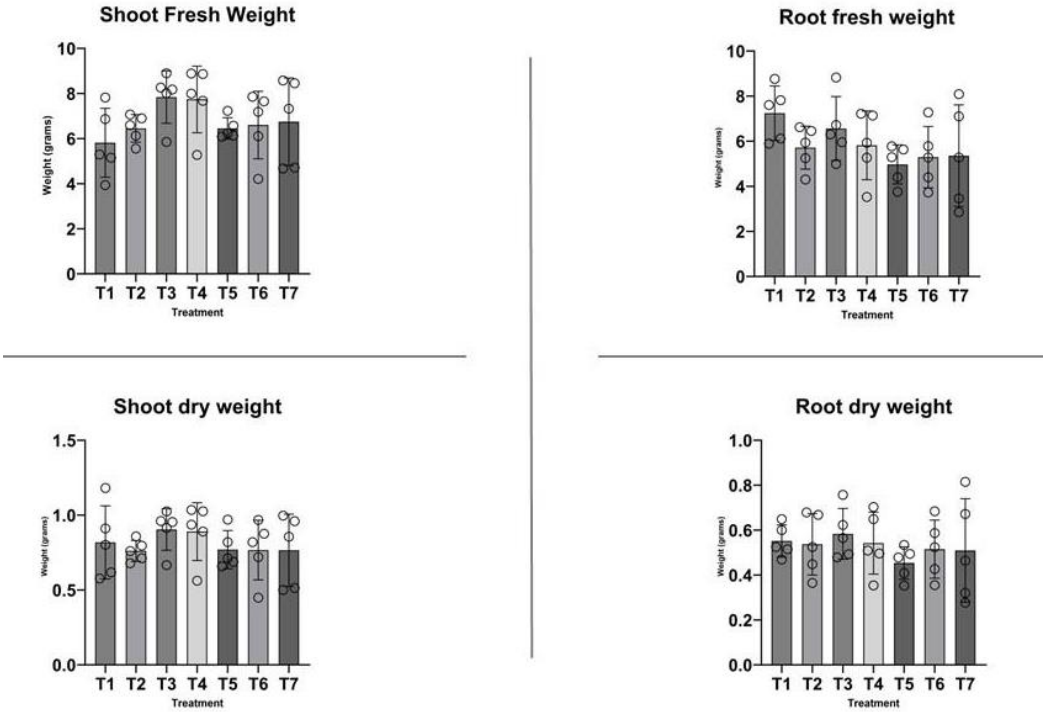
Appendix 1.4: **Graph showing the results from our endo-colonization experiment subjecting green pea to nitrogen stress.** The table shows clearly no growths on almost all plates showing that during nitrogen stress *B. mycoides* M2E15 is still excluded from endo-colonizing green pea.



Appendix 1.5: **Graph showing the plant weight parameters from our nitrogen stress experiment.** The graph shows a clear difference between the plant parameter shoot length under different nitrogen conditions while not showing a difference between *B. mycoides* treatments.



Appendix 1.6: **Graph showing the plant parameter Shoot length from our nodulation experiment.** The graph shows no significant difference between the different treatments.



Appendix 1.7: **Graph showing the plant weight parameter from our nodulation experiment.** The graph shows no significant difference between the different treatments.