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Article

# Investigating the role of biotic factors in host responses to rhizobia in the system *Medicago truncatula*

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## SUMMARY

**Nitrogen-fixing bacteria, such as the legume mutualist rhizobia, convert atmospheric nitrogen into a form that is usable by living organisms. Leguminous plants, like the model species *Medicago truncatula*, directly benefit from this process by forming a symbiotic relationship with rhizobia. Rhizobia fix atmospheric nitrogen to a bioavailable form for the plant and are supplied with photosynthetically fixed carbon in return. Host plants have the ability to dynamically respond to various biotic factors in their respective environments. In the context of rhizobial associations, hosts have the ability to regulate resources based on the bacteria's nitrogen fixing abilities as well as external factors such as drought and salinity. We investigated how *M. truncatula* responds to non-rhizobial bacterial partners by measuring the growth of the plant, nodulation on the roots, rhizobial fitness, and nutrient allocation to ineffective rhizobial partners within the nodules. We found that *Burkholderia* spp. inhibited the growth of the nitrogen fixing bacteria *Ensifer meliloti*, Rm 1021. Even though *Burkholderia* inhibited rhizobia growth, *M. truncatula* showed the increased growth when inoculated with *Burkholderia* and Rm 1021. These data suggest that there may be a tripartite interaction among *Burkholderia*, Rm 1021, and *M. truncatula*.**

## INTRODUCTION

A microbiome is a community of microbial organisms living in close association with a multicellular organism. The plant microbiome is generally thought to be composed of three parts: the endosphere, which contains microbial organisms living throughout host tissue; the phyllosphere, where bacteria live on foliar leaf surfaces; and the rhizosphere, in which microbes live on the surface of plant roots (1,2). A plethora of bacterial partners exist in symbiosis with plants (3).

Legumes are flowering plants that belong to the family Fabaceae, a family containing many agriculturally important crops like the soybean *Glycine max* and the common bean *Phaseolus vulgaris*. Legumes are particularly interesting because they are able to form a specialized, mutualistic relationship with a group of nitrogen fixing bacteria. Nitrogen fixing bacteria, such as legume symbionts, and lightning are the only common natural phenomena that convert atmospheric nitrogen (N2) into ammonia (NH3), which is a form of nitrogen that is usable by plants and most other living organisms (4). Biological nitrogen fixation is a process in which rhizobia fix atmospheric nitrogen to a bioavailable form. A symbiotic relationship forms as bioavailable nitrogen is supplied to the legume, and rhizobia are supplied with photosynthetically fixed carbon in return (5). This work focuses on understanding interactions within the rich microbial diversity of the rhizosphere (6).

A variety of processes occur in this region including various forms of signaling, secretion, interactions with pathogens, nitrogen fixation, interactions with compounds such as flavonoids, and nodulation formation (7,8). Rhizobia are bacteria that specifically fix nitrogen when associated as an endosymbiont (9). A nitrogenous complex is activated when rhizobia settle in a plant-derived structure called a nodule. This begins when a plant sends out signals such as flavonoids, which attract rhizobia to the roots of the legume (10). The rhizobia attach to hooked root hairs, which form small structures along the root of the plant called nodules (11). Between five and twenty percent of the photosynthetic products from photosynthesis are directed to the rhizosphere, demonstrating the importance of this exchange (12).

In addition to rhizobia, other types of bacteria can be found in the microbiome. Bacteria that are not rhizobia cannot interact with the plant to form nodules individually, meaning that nodules cannot contain only non-rhizobial partners without any rhizobia present. However, nodules can house rhizobia and non-rhizobial bacterial partners simultaneously, as bacteria that are near rhizobia can be included in the nodule with the rhizobia during the infection process (13). This raises a variety of questions about what influence the presence of these non-rhizobial bacterial partners has on traits such as the legume's growth and nodulation, the fitness of the rhizobial symbiont, and the host plant's ability to limit the growth of less effective bacterial partners.

However, microbes have been found to "cheat," as they do not fix nitrogen but still claim the resources from the host plant. As a result, host plants have evolved the ability to delegate resources to rhizobia based on their nitrogen fixing abilities (14). This mechanism is referred to as sanctions, and it functions to limit the growth of ineffective rhizobial partners. This research sheds light onto the mechanism of sanctions and examines the relationship between biotic factors in the plant microbiome and plant health and productivity.

Furthermore, understanding how a plant obtains

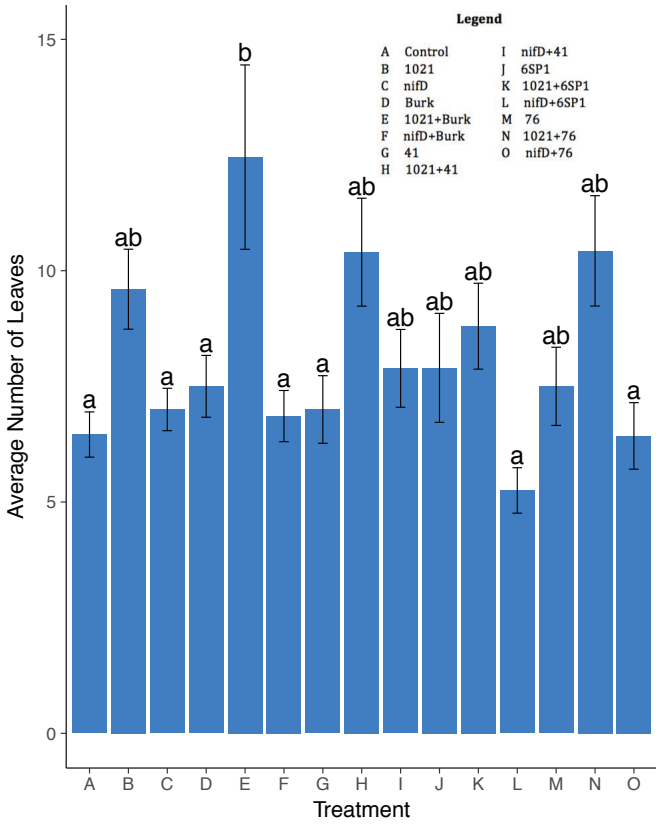


**Figure 1: Average Number of Leaves per Treatment.** The number of leaves is a direct measurement of the growth of *M. truncatula*. If a plant has more leaves, then it can be assumed that it is acquiring nutrients at a high rate due to the presence of the bacteria from the inoculations. From the analysis of variance test shown in Figure 1, the only treatment that is statistically different from the rest is Treatment E, or Rm 1021 + *Burkholderia*.

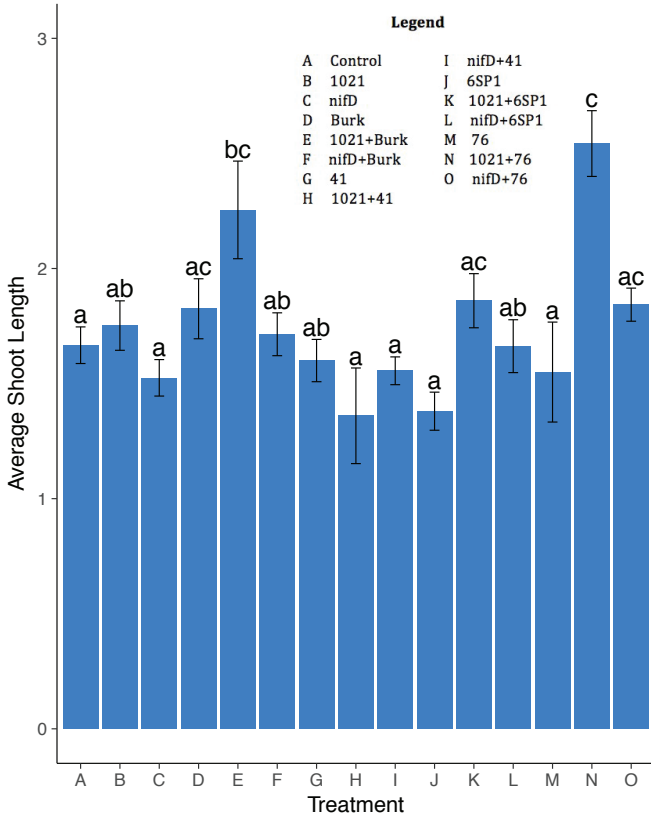


**Figure 2: Average Shoot Length per Treatment.** The shoot length is also a direct measurement of *M. truncatula*'s growth. From the analysis of variance trust shown in Figure 2, there are two statistically significant treatments: E (Rm 1021 + *Burkholderia*) and N (Rm 1021 + 76). *M. truncatula* seems to be acquiring nutrients more easily in the presence of these bacterial combinations.

nitrogen is essential to combating environmental problems caused by anthropogenic activities. Nitrogen is one of the biggest agriculturally limiting nutrients in modern society, and the only solution we currently have is the Haber-Bosch process, also known as industrial nitrogen fixation (16). In this process, fossil fuels are burned to produce an immense amount of energy to break the triple bond in atmospheric nitrogen to generate ammonia/ammonium, which is then applied to agricultural fields. However, the process of adding nitrogenous fertilizers to land produces greenhouse gases and causes the excess nitrogen to leach into water sources because plants only absorb around 20% of the nitrogen from fertilizers applied to fields (17). This influx of nitrogen causes harmful algae blooms that kill aquatic life and dramatically alter the chemistry of nearby water sources (18). Additionally, the addition of nitrogen fertilizers disrupts native microbial communities either by decreasing natural nitrogen fixation or by increasing the prevalence of methane-producing bacteria (19). Understanding how a host plant optimizes its resource acquisition and how external factors influence the symbiotic relationship can allow us to develop methods that promote the activities that allow for improved plant fitness and, by extension, yields. Understanding biological nitrogen fixation and how hosts are able to maintain stable cooperative relationships has the potential to enable us to better predict what environmental factors could lead to mutualism breakdowns, further optimize the current symbiotic relationships, and even transfer the symbiosis to non-leguminous host plants (20).

This research focuses on the leguminous plant species *Medicago truncatula* and investigates how bacterial partners influence *M. truncatula*'s ability to form symbiotic relationships with rhizobia that are housed in nodules. The approaches taken in this research are unique as we account for a tripartite interaction between the host legume, rhizobial partners, and non-rhizobial bacterial partners, and we analyze the influences of non-rhizobial bacterial partners on nodulation, rhizobial fitness, plant growth, and the sanctioning of ineffective rhizobial partners. We hypothesize that the presence of plant-associated bacteria, in addition to rhizobia, will result in improved plant fitness relative to rhizobial inoculations alone. Investigating the interactions between the host plant and the bacteria is valuable for developing sustainable agricultural processes, informing environmental concerns such as greenhouse emissions, and understanding the role of microbes in biogeochemical cycles (15).

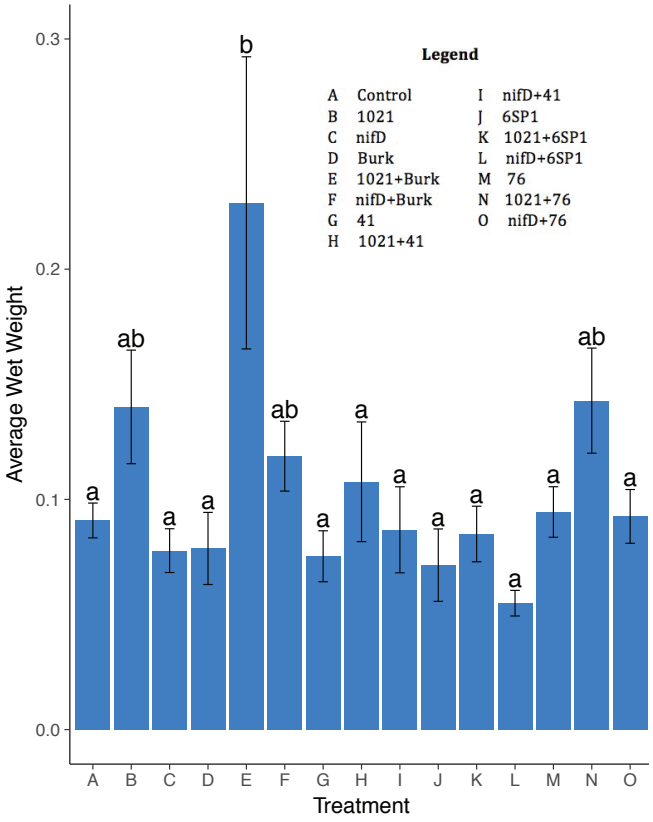


**Figure 3: Average Wet Weight per Treatment.** Figure 3 shows the average wet weights per treatment plotted on graphs. The analysis of variances test showed us that Treatment E (Rm 1021 + *Burkholderia*) seems to have induced the highest growth in *M. truncatula*.

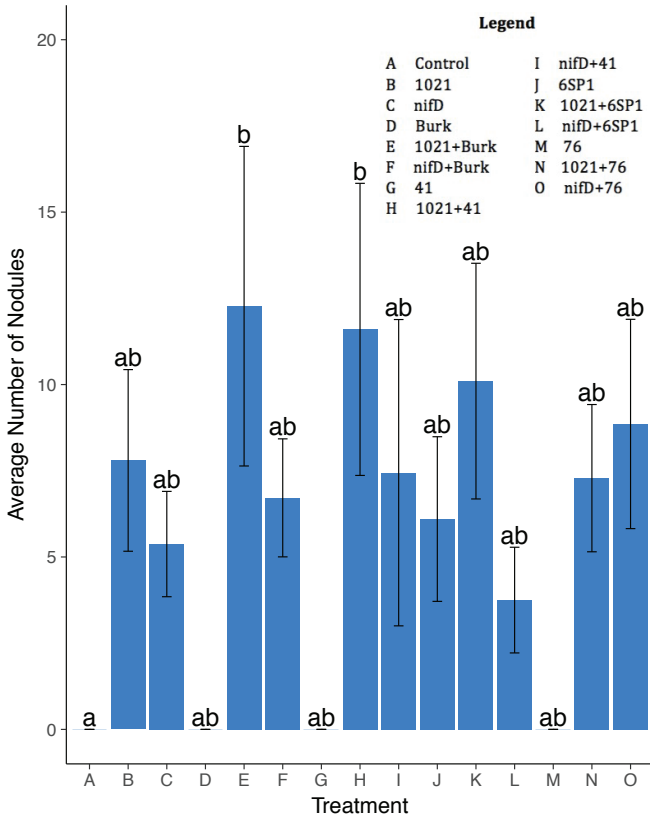


**Figure 4: Average Number of Nodules per Treatment.** Figure 4 shows the average number of nodules plotted for each treatment. The analysis of variance test shows that treatments E (Rm 1021 + *Burkholderia*) and H (Rm 1021 + 41) are statistically different from the rest.

## RESULTS

We recorded a variety of growth measurements for *M. truncatula* to determine the influence of non-rhizobial bacterial partners on host responses to rhizobia. The growth measurements included the average number of leaves per treatment, average shoot length per treatment, average wet weight per treatment, average number of nodules per treatment, and average colony forming units per treatment.

### Co-Inoculation of Rm 1021 + *Burkholderia* promotes growth of *M. truncatula*

*M. truncatula* demonstrated the highest metrics of growth when co-inoculated with a rhizobial and a non-rhizobial bacterial partner, specifically the treatment of Rm 1021 + *Burkholderia*. To reach this conclusion, we collected statistical support. First, we plotted the averages of all treatments for each of the five different types of growth metrics on a graph (**Figures 1-4**). We then applied error bars and a one-way ANOVA test to determine any significant differences in the averages of each treatment for each of the different metrics. Treatment E, or Rm 1021 + *Burkholderia*, was the only treatment resulting in a significant difference, as it was the only treatment to be assigned a different letter from all the other graphs in all the measurement graphs shown in **Figures 1-4**. The ANOVA and Tukey test showed a significant difference in Rm 1021 + *Burkholderia* for the average number of leaves, average shoot length, average wet weight, and average number of nodules. We chose these measurements as they provide direct and quantifiable insight into the physical growth of the plant, allowing us to easily assess the influence of the bacteria on the legume's growth.

### *Burkholderia* inhibits the growth of Rm 1021

The growth inhibition assay provides insight into how the rhizobia and isolates are interacting with each other when they are not spatially limited (which they are in a nodule) given the abundant space on the petri dish. Plating the bacteria on media so that it becomes visible to the human eye allowed us to easily describe what is physically occurring in the nodule, as we are not able to view inhibition if the bacteria are still inside the nodule. The only plates that showed inhibition were the plates with disks soaked in the *Burkholderia* isolate that initially had Rm 1021 spread onto them. We uploaded pictures of these plates (**Figure 5**) to the ImageJ computer program that provided measurements of the size of inhibition on the plates. We normalized this to the size of the petri dish and calculated that the average size of inhibition was 0.254 ± 0.006 cm. Every other isolate and control plate showed

zero inhibition. This data shows that *Burkholderia* can inhibit the growth of the rhizobial species Rm 1021, as the zones of inhibited growth are significantly different from the control plate, which had no inhibition.

## DISCUSSION

There are two major pieces of data to consider from this experiment. The treatment of Rm 1021 + *Burkholderia* promotes the most growth in *M. truncatula*, and *Burkholderia* seems to inhibit the growth of the nitrogen fixing rhizobia Rm 1021. The ability of *Burkholderia* to simultaneously promote the growth of *M. truncatula* while inhibiting the growth of the nitrogen fixing Rm 1021 may seem opposing, but instead provides the opportunity to offer closer analysis on the intricacies of the relationship between the rhizobial species, the isolate, and the host legume. Additionally, the signs of inhibition of growth on a TY agar plate from the growth inhibition assay can be contextualized to the nodule and provide further explanations for how the presence of non-rhizobial bacterial partners alter host responses to rhizobia.

These data raise a variety of conclusions. Even though *Burkholderia* inhibits the growth of nitrogen fixing rhizobia (**Figure 5**), *M. truncatula* showed the most positive trends of growth when inoculated with *Burkholderia* and Rm 1021 (**Figures 1-4**). This is the opposite of what is expected, since the amount of nitrogen that the plant would be receiving would theoretically be less since the growth of the nitrogen fixing rhizobia is inhibited. However, it appears that the plant growth is not wholly dependent on the proportion of Rm 1021, and the presence of *Burkholderia* improves plant growth while simultaneously inhibiting the growth of Rm 1021. This suggests that there potentially is a tripartite interaction between *Burkholderia*, Rm 1021, and *M. truncatula*. The ineffective mutant nifD was included in this experiment as some of the theories regarding sanctions overlap with how plants regulate pathogenic/less beneficial bacteria. Therefore, including nifD served as an interesting built-in reference point. However, the data offered no significant trends on how responses to ineffective rhizobia were altered due to unexpected variance that was introduced to the plants that were inoculated with the ineffective mutant nifD. It would be both interesting and important to repeat the above research using nifD and other ineffective mutants to determine if *Burkholderia* alters the host's ability to sanction ineffective partners.

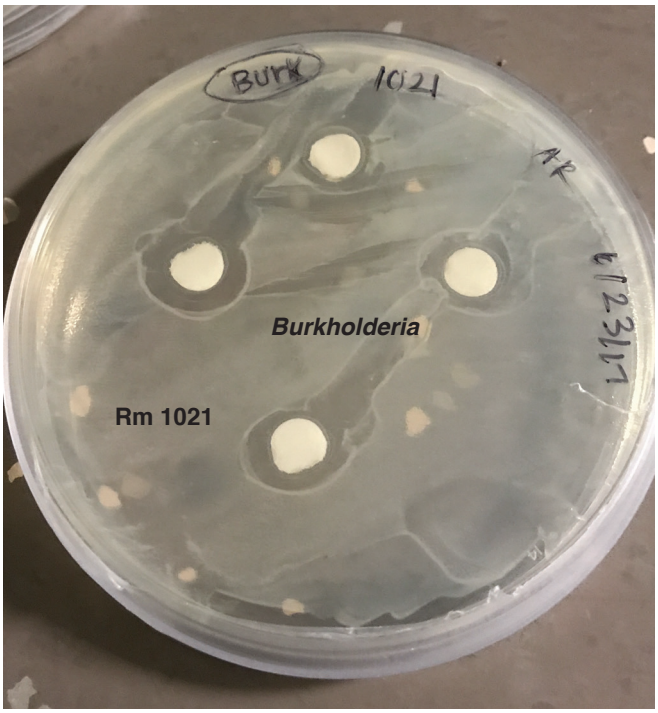


**Figure 5: Growth Inhibition of Rm 1021 Caused by *Burkholderia*** This plate first had the liquid culture of Rm 1021 spread onto it. After it dried, sterile disks soaked in the supernatant of *Burkholderia* were placed on the agar. This plate demonstrates clear zones of inhibition around each of the four disks, most likely caused by some type of unknown antimicrobial compound. The length was measured of each zone of inhibition around each disk. The average size of inhibition was 0.254 ± 0.006 cm. This data shows that *Burkholderia* can inhibit the growth of the rhizobial species Rm 1021.

Our hypothesis, which states that the presence of plant-associated bacteria results in improved plant fitness relative to rhizobial inoculations alone, is supported by the data from this research. Even though the data does not show that rhizobial fitness was improved, the possible influences of the tripartite interaction agree with the hypothesis. Additionally, the results also agree with previously published research. There are numerous microbes in the soil that result in improved plant growth without fixing nitrogen; these microbes are known as plant growth promoting rhizobacteria (21). However, this research is investigating this growth promotion in the host when the host plant is specifically in symbiosis with rhizobia (Rm 1021), with future aims to see how plant growth promoting rhizobacteria alter the host's physiological responses to rhizobia through the sanctioning mechanism.

Some external factors may have introduced additional variance to the collected data, including altered environmental conditions as a result of unpredicted growth facility changes. Additionally, some plants were exposed to external stressors. When leguminous plants are exposed to stress they will divert energy from nodules to other processes like reproduction (11). This explains why in the stressed out plants, there were very few or even no nodules, as the nodules fell off the roots since they could not sustain themselves. This introduces a lot of variance in the graph showing the average number of nodules and can obscure trends that may be present. There are possible improvements that could be applied to these techniques, including using a better surface sterilization technique and using a flow cytometer to record data in a more quantitative fashion.

There are many promising future directions that can be pursued from the presented work. There are 89 isolated strains collected from *M. truncatula* and *M. lupulina* in the field that can provide a better understanding of how these rhizobial and non-rhizobial bacterial partners influence the regulatory

mechanisms. Additionally, the growth inhibition assay can be performed using more of these strains to provide quantitative data of differences in the ability of microbes to restrict the growth of other microbes. This assay can be taken a step further by using a mass spectrophotometric technique to see how certain microbes inhibit the growth of other microbes by determining the antimicrobial compound the growth inhibiting organism is releasing and what impact this would have in the nodule when these microbes are co-existing.

Other types of data can be collected that may further support the conclusions made earlier and provide further explanations regarding the tripartite interaction. Growth curve data could be collected to compare the rates of growth of the rhizobia and isolates. This can be done by collecting optical density 600 readings by using a Hybrid Plate Reader to take readings of solutions of the microbes that are normalized to a set concentration. This would supplement the growth inhibition data, as this would represent the environment in which the microbes are spatially limited. Readings could first be collected for solutions with each of the isolates and rhizobia alone, and then co-inoculations could be done to see which of the bacteria are out-competing the other.

Current agricultural practices have dramatically altered native microbial species by disrupting the niche of diazotrophs and rhizobia. The data from this project is important to understand how biological nitrogen fixation operates when the host plant is introduced to non-rhizobial bacterial partners. This research is also an important step in the development of sustainable agricultural practices. We need to understand the role of the rhizosphere and endosphere communities in soil environments and how they influence nitrogen fixation so that we can promote the activities and environments that allow legumes to optimize their nitrogen intake. With this knowledge, we will be able to start to become less dependent on the Haber-Bosch process. This is important as the world population is rising exponentially, and we will run out of the resources we need to survive without sustainable practices.

This research sheds light on how various non-pathogenic bacterial species that live in association with host leguminous plants alter host responses to rhizobia. While the discovery of a possible tripartite interaction may not fully explain all the factors that can influence the host's growth promotion and the sanctioning mechanism, it serves as a first step to investigating and understanding the relevance of the rhizosphere and endosphere communities in soil environments. Modern agricultural practices have dramatically altered microbial soil communities, and the consequences of these changes remain vastly understudied. Continuing this work and related work will potentially allow researchers and agriculturalists to develop and implement sustainable cropping systems that take into account the importance of native microbial communities.

## MATERIALS AND METHODS

### Bacterial Culture Preparation

Bacterial cultures were prepared for both the rhizobial and non-rhizobial bacterial species. Sterile tryptone yeast (TY) agar media was made and poured onto Fisherbrand petri dishes. TY agar contains 6.0 g tryptone yeast, 3.0 g yeast extract, 0.38 g CaCl2, and 16.0 g bacto-agar per 1000 mL. Samples from bacterial freezer stocks of each isolate were streaked across the surface of the agar of their respective plates. These stocks were isolated from the rhizosphere of wild growing *M. truncatula* and *Medicago lupulina* and were stored at -80°C in a 50% glycerol solution. The plates were then sealed with parafilm. The plates were placed in an incubator set at 30° C.

The bacteria grew on TY plates in the incubator for two days. Liquid cultures were started using sterile 15 mL conical tubes filled with 10 mL of sterile TY broth. TY broth is prepared as described above, minus the addition of agar. Sterile pipette tips were used to transfer individual colonies to the 15 mL conical tubes. The tubes were sealed using micropore tape, which allows oxygen to pass through the seal. The tubes were placed in an incubator at 30°C and set to shake at 160 rpm. Liquid cultures were left to grow for four days before use.

### Plant Growth

In order to closely inspect the interactions between rhizobial and non-rhizobial bacterial partners and investigate the sanctioning mechanism, we collected data about nodules that formed when plants were exposed to different types of bacteria. In order to accurately compare the impacts of different bacteria on plant growth and nodulation, we grew plants using a method that ensured maximum sterility, such that the plants were only exposed to bacteria that was pipetted from the liquid cultures.

Plants were grown in medium sized cone-shaped containers referred to as "conetainers." Pieces of rope approximately four inches in length were cut, knotted, wrapped in a paper towel, and threaded through the bottom of the conetainer to serve as a wick. The wick was connected to a reservoir that contained water. Each conetainer was then filled to the top with sterile vermiculite, which is a substrate that can be used for sterile or semi-sterile plant growth. All of the conetainers were autoclaved, 30 mL of water was added to each of the conetainers, and they were autoclaved a second time. After the second autoclave, 30 mL of 1x Fahraeus nutrient solution was added to each of the conetainers, and the conetainers were autoclaved for a final time. Fahraeus solution is 0.5 mM MgSO4 • 7H2O, 0.7 mM KH2PO4, 0.8 mM Na2HPO4 • 2H2O, 50 mM Fe-EDTA, 0.1 µg/L MnSO4, 0.1 µg/L CuSO4, 0.1 µg/L ZnSO4, 0.1 µg/L H3BO3, 0.1 µg/L Na2MoO4.

### Seed Sterilization and Germination

A17 cultivar *M. truncatula* pods were collected from bulked lab plants. The pods were crushed and scarified on 600 grit sandpaper. Seeds were sterilized in a 5% bleach solution for 2 minutes then rinsed 6 times using sterile water.

The seeds were then imbibed in water overnight at 4° C. Seeds were transferred to petri dishes, which were placed in a dark cabinet to allow for germination. The seeds were left in the germination cabinet for two days before planting.

## Bacterial Inoculations

The research group has previously isolated 89 strains of bacteria from the rhizospheres and endospheres of native legumes *M. truncatula* and *M. lupulina* in an effort to better understand how legumes shape their microbial communities. Here, we present work that focuses on a random subset of these isolates as an early stage of large data collection efforts. A set inoculation treatment pattern was used: control, Rm 1021 (Ensifer meliloti), nifD (ineffective mutant), isolate, isolate co-inoculated with Rm 1021, and isolate co-inoculated with nifD.

The first step of making the inoculants was starting liquid cultures as explained previously. 1 mL of each liquid culture was pipetted into a sterile eppendorf tube. The tubes were spun in a centrifuge for 10 minutes at 9000 rpm, the supernatant was removed, and the remaining pellet was resuspended in 1/2x Sodium Phosphate Buffer (PBS). This step was repeated again to ensure that only the bacteria were present in the solution. 1:20 dilutions were made in 1/2x PBS in Eppendorf tubes to ensure that concentration readings fell in the linear range of a spectrophotometer. The concentrations of these cultures were checked using a DU 800 Spectrophotometer, with 1/2x PBS as the blank. The equation M1V1 = M2V2 was used to find the volume of the culture that needed to be diluted in 1/2x PBS to create the final inoculant. A concentration factor of 20 was used to account for the 1:20 dilution earlier and the final concentration was 1x106, and the final volume was 50 mL. Once the volume needed from the original culture was determined, that volume was pipetted out from 50 mL of 1/2x PBS in a conical tube and then the calculated volume of culture was pipetted in to create the inoculant. 1 mL of each inoculant was then pipetted onto the surface of the vermiculite for the plants in each treatment. The plants grew for 2 weeks before being inoculated and then grew for another 4 weeks before they were harvested.

## Harvesting

Harvesting consisted of three major steps: collecting data for plant growth traits, sterilizing root systems, and crushing nodules to perform serial dilutions to get the colony forming units (CFU) count. Shoot length and leaf number were the first data collected. The vermiculite from the conetainer was dumped into a biohazard bag. Vermiculite was rubbed off the plant, and the plant was placed in deionized water to rinse off excess vermiculite. The number of nodules was counted by looking closely at the root systems. The plant was dried off using a paper towel, and the wet weight of the plant was recorded using an analytical balance. The plant was placed in a Ziploc bag, and milli-Q water was added to the bag. The Ziploc bags were then left in a cold room at 4°C until they were ready to be sterilized. The plants were transferred to conical tubes, and 5% bleach solution was added to sterilize the plants. The conical tubes were shaken, and the bleach was removed after 2 minutes. The plants were then rinsed with deionized water 6 times, and the roots were considered sterile.

Flat-bottomed 96 well plates were used to crush nodules. 180 µL of a 1:1 solution of 1/2x PBS and glycerol was added to all of the wells. A small root section and 3 nodules were taken from each plant. If a plant did not have nodules, 4 root small sections were taken instead. Each plate was divided into 6 sections so that 6 plants could be used per plate. For each sample, three serial dilutions were performed. Using sterile forceps, the root sections and nodules were placed into different wells of the plate, and a sterile multicrusher was used to crush the samples so the bacteria were released into the solution in the well. 20 µL of the initial solution was then pipetted to the next column and so on until the fourth column had a concentration of 10-3. A 4x4 grid was then drawn on a TY plate and 10 µL from each well was pipetted onto the TY plate in each section of the grid. The plates were left to dry and then were paraflimed and placed in the 30° C incubator for 2-3 days until the CFUs from each of the dilutions could be counted.

## Growth Inhibition Assay

To investigate the interactions between rhizobial and non-rhizobial bacterial partners in the absence of a host plant, a growth inhibition assay was conducted.

Rhizobia and isolate liquid cultures were started (**Table 1**). TY agar was made and poured onto petri dishes. 300 µl of the rhizobial species Rm 1021 and WSM were pipetted onto multiple separate dry TY plates. The cultures were spread

| Isolate ID | Host Species | Category | Bacterial Species |
|---|---|---|---|
| *Burkholderia* | *M. truncatula* | Endosphere | *Burkholderia spp.* |
| 41 | *M. truncatula* | Endosphere | *Paenibacillus sp.* 4-21 16S ribosomal RNA gene, partial sequence |
| 6SP1 | *M. lupulina* | Endosphere | *Enterobacter cloacae* strain XJ31 16S ribosomal RNA gene, partial sequence |
| 76 | *M. truncatula* | Endosphere | *Pseudomonas sp.* D9 16S ribosomal RNA gene, partial sequence |

**Table 1:** Identification of Isolates Used for Inoculation

onto their respective plates evenly along the surface of the agar using sterile tools, and the plates were set aside to dry. Small disks were cut from filter paper and autoclaved. The supernatant of each isolate as well as sterile media was collected in a sterile conical tube using a GS 0.22 µM filter attached to a sterile syringe. The disks were soaked in the supernatant of each isolate or in the control media, and were placed on the Rm 1021 and WSM plates using sterile forceps. Four disks were placed on each plate and this was completed in triplicate for each isolate and for the media control plates. The plates were set aside to dry then parafilmed and placed in the incubator set at 30°C. The plates were checked every day for a week to see if there were any zones of inhibition caused by the isolates.

### Statistical Analysis

Graphs were made using R (version 3.4.1) by plotting the averages of the four different growth measurements. Error bars and an analysis of variance test (ANOVA) were applied to the graphs to determine if any of the treatments were significantly different. A Tukey test was used in conjunction with an ANOVA to assign a letter or combination of letters to each bar graph as a means to compare all the bars to each other. The bars with the same letters above them are not statistically different from each other, while bars with different letters are statistically distinct from each other. Significance was determined by p-values $< 0.05$. A program was also used to calculate the size of the zone of growth inhibition on the *Burkholderia* and Rm 1021 plate.

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