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The role of Sinorhizobium meliloti Type IVb pili protein on legume nodule morphology and infection

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The role of *Sinorhizobium meliloti* Type IVb pili protein on legume nodule morphology and infection

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Abstract

The symbiotic relationship between legumes and rhizobial soil bacteria leads to the conversion of nitrogen gas to biologically available forms of nitrogen. A critical step in this interaction is bacterial attachment to the host root. A variety of factors are involved in attachment, such as the Type IV pili (Tfp). In pathogens, Tfpb mediates tight adherence to host cells, promoting colonization, however in symbiotic interactions, the role of Tfpb–encoded by *pilA* genes–is not well understood. To assess the role of Tfpb pili during symbiosis, *Melilotus alba* plants were inoculated with wild type *Sinorhizobium meliloti* or *ΔpilA* deletion mutants; roots were harvested at different stages of development. Plants inoculated with *ΔpilA* mutants showed abnormalities in root nodule development, including patchy infection patterns, irregularly shaped nodules, and clusters of partially infected or uninfected nodules. However, when evaluating plant growth overall, the plants infected with *ΔpilA* mutants looked comparable, if not healthier than the wild type controls, indicating that the nodule abnormalities caused by the *pilA* deficiency might be beneficial; the nodule clusters might ultimately accommodate more bacteria to fix nitrogen. Expression of genes associated with plant defense and symbiosis was also evaluated. Preliminary results indicate that plants inoculated with *ΔpilA* mutants have reduced expression of *DNF2*, a gene that is necessary for the development and maintenance of nodules.

Introduction

Nitrogen is one of the most important but also most limiting nutrients for plants. While nitrogen is readily available as a gas, atmospheric nitrogen cannot be used by plants. Chemical fertilizers have become popular as a method for combatting nitrogen-limiting environments, especially in agricultural settings, however, chemical fertilizers have a multitude of ramifications including their effects on the environment. Runoff from chemical nitrogen fertilizers often results in nitrogen pollution, causing eutrophication (Howarth, 2007). Soil bacteria called rhizobia are diazotrophic and can convert atmospheric nitrogen into biologically available forms, both as free-living bacteria and in symbiosis with plants (Kuykendall et al., 2015). The process of fixing nitrogen is an extremely energetically costly process and is thus only carried out in nitrogen limiting environmental conditions (Mylona et al., 1995). Symbiotic nitrogen fixation is also much more efficient than that of free-living bacteria because the bacteria are housed in specialized root structures in the plant and are provided with a carbon source from the plant in exchange for the fixed nitrogen (Olroyd et al., 2011). This symbiotic interaction has become of interest to researchers because of its potential for increasing nutrient, specifically nitrogen, acquisition in plants.

Rhizobia engage in a variety of symbioses, such as the alpha (α) Rhizobium-legume interaction. For this interaction to successfully occur, both infection and nodule development in the host roots must occur. Communication between symbiotic partners begins in nitrogen-poor soils with the emission of species-specific flavonoids from the roots of the host-plant. Rhizobia in the soil recognize and respond to the flavonoids through chemotaxis towards the higher

flavonoid concentrations. The flavonoids also induce the expression of *nod* genes that encode enzymes for the biosynthesis of the host specific Nod Factor signal that is then emitted by the bacteria. One of the responses from the plant upon the reception of Nod Factor is the deformation of the root hair into a shape called a shepherd's crook, which is classified by the end of the root hair curling in on itself. When the root hair deforms into this shape, it traps the bacteria that have attached to the root hair. The bacteria then divide and grow into the root hair of the plant through an infection thread. The bacteria travel into the plant and are released into the cytoplasm of cortical cells. Nod Factor also induces divisions of cortex cells to form the nodule primordium (Eckardt, 2006).

After the bacteria have been released into the plant cells, they develop into bacteroids that are specialized for synthesis of the nitrogenase enzyme that is necessary for the conversion of atmospheric nitrogen into biologically available for of nitrogen through nitrogen fixation. Nitrogenase is only effective in anoxygenic conditions, so the plant produces leghemoglobin to bind to oxygen and remove it from the nodule (Puppo et al., 1984). Healthy nodules have a pink color due to the presence of leghemoglobin and this coloration is an indicator that nitrogen fixation is efficiently occurring. Uninfected nodules or non-nitrogen fixing nodules are white because of the absence of the oxygen-binding leghemoglobin; when no nitrogen fixation is occurring, no leghemoglobin will be produced (Puppo et al., 1984).

Non-nitrogen fixing nodules are often observed to exhibit an abnormal brown color that is indicative of phenolic compounds accumulation, a response that is associated with plant defense (Bhattacharya, 2010). A plant's defense response to infection from pathogens is multifaceted and includes the accumulation of phenolic defensive compounds localized in the infected area. The phenolic compound accumulation that has been found in nodules that do not

fix nitrogen suggests that when nitrogen fixation does not effectively occur, a defense response is elicited to remove the ineffective bacteria. Inability to fix nitrogen has also been observed to result in the upregulation of genes such as *PR10* that are also associated with plant defense responses (Samac et al., 2010).

The Rhizobium-legume symbiosis is mediated by many genes and genetic pathways, including those associated with plant defense. Other genes that are involved in the symbiosis have been identified to be necessary for the normal development and maintenance of nodules. Downregulation of such genes has been observed to result in early nodule senescence, phenolic compound accumulation, abnormal nodule development, patchy infection patterns and necrotic nodules, which are phenotypes that are also consistent with those induced when nodules are not fixing nitrogen. Examples of such genes include *DNF2* and *NAD1* (Berrabah et al., 2015) (Domonkos et al., 2017). *DNF2* is also involved in the suppression of an immune response from the host plant. Immune responses are suppressed during symbiotic interactions to allow for the colonization of the plant by the symbiotic partner, in comparison to the triggering of immune responses that occurs during interactions with pathogenic organisms that the plant aims to get rid of. Some phenotypes that are associated with downregulation of *DNF2*, such as phenolic compound accumulation, can be attributed to the restoration of the plant's defense response (Berrabah et al., 2015).

Another factor involved in the symbiosis is the pili protein encoded by *pilA* genes. The pili protein is a bacterial surface protein that is necessary for motility and attachment to the host (Hirsch et al., 2008) (Zatakia et al., 2014). The role of the protein is much more well-understood in pathogenic interactions than in symbioses. Localization studies on $pilA_2$ indicate that the gene is active in bacteria through the infection thread, after the initial attachment, suggesting that the

pilA genes are more involved in the symbiosis than was previously thought. Previous research has also concluded that bacterial deletion mutants for *pilA* genes exhibit inconsistent infection patterns in nodules and induce abnormal nodule development in host plants.

The goal of this study was to further evaluate the role of the pili protein in symbiosis by observing the effects of *Sinorhizobium meliloti* $\Delta pilA_1A_2$, and $\Delta pilA_1A_2A_3$ deletion mutants. We confirmed that *pilA* deletion mutants induce abnormal nodule development and patchy infection patterns. Plants inoculated with mutant bacteria also appeared to exhibit accumulatin of phenolic compounds. Despite abnormal infection and nodule structure, *ΔpilA* mutants did not result in a significant difference in health or growth patterns of the plants. The mutant bacteria did, however, induce downregulation of the symbiosis related gene *DNF2.* The downregulation of *DNF2* and accumulation of phenolic compounds suggest that the mutant bacteria are inducing a defensive response in the plants.

Materials and Methods

Bacterial culturing

Sinorhizbium meliloti cultures were prepared by streaking frozen stocks of Rm 1021, *ΔpilA$_1$A$_2$*, and *ΔpilA$_1$A$_2$A$_3$* onto TY agar plates (Beringer, 1974) with streptomycin antibiotics and incubated at 30°C overnight. 5 mL of liquid TY with streptomycin and tetracycline were inoculated with single colonies from the plates and grown for 48 hours at 30°C and 200 rpm. The cultures were then diluted to an OD600 concentration of 0.2 AU with RDM (Vincent, 1970) and the cells were resuspended in Hoagland's medium without nitrogen (Hoagland & Arnon, 1938). Each magenta jar was inoculated with 1 mL of inoculant 1 week after the seeds were planted. The following concentrations of antibiotics were used: streptomycin at 100 μg/mL and tetracycline at 10 μg/mL.

Plant growth and inoculation

Magenta jars were filled with a 3:1 ratio of vermiculite:perlite, watered with Hoagland's medium, and sterilized by autoclave. One control group was watered with Hoagland's medium complete while the rest of the groups were watered with Hoagland's -N. *Melilotus alba* seeds were sterilized with 95% v/v ethanol for 5 minutes, 5.25% (v/v) sodium hypochlorite for 20-30 minutes. Seeds were rinsed with sterile 5 times with sterile water to remove bleach and planted in the sterile Magenta jars. 10 seeds were planted per container. The plants were grown in a growth chamber at (add temp). Following growth for one week, plants were inoculated; the -N control group was left inoculated and the three experimental groups were inoculated with either *S. meliloti* Rm 1021 (WT), *ΔpilA$_1$A$_2$* mutant, or *ΔpilA$_1$A$_2$A$_3$* mutant.

Tissue harvesting and plant growth assay

The plants were harvested one-, two- and three-weeks post-inoculation (WPI) or six months post inoculation. The one-, two- and three-WPI plants were carefully removed from the vermiculite-perlite mixture and the roots were severed from the stem. The roots were observed under a stereoscope and light microscope. Both the root and shoot tissues were stored in either RNAlater solution or fixed for histology. The six-month-old tissues were also carefully removed from the planting media and the whole plant was weighed. One set of plants was dried in an incubator at 65°C for two days and dry mass was measurements were taken. The two other sets of plants were cut between the root and shoot. The roots were observed and all the tissues were again stored in RNAlater or fixed for histology. One-way ANOVA tests were run on the biomass data.

Histological Studies

S. meliloti strains constitutively expressed the *gusA* reporter gene. The root tissues were stained with X-Gluc staining solution which allows for visualization of the bacteria by coloring the bacteria blue (Jefferson et al., 1987). Stained tissues were observed under and light microscope and infection rate data was collected (total number of nodules per root and numbers of nodules that were uninfected, partially, or fully infected. Nodules were severed from the root and the nodule tissues were treated with a graded ethanol series from 50% to 100% ethanol, then a graded ethanol to tertiary butyl alcohol (TBA) series, and finally a graded TBA to paraffin wax series. Once embedded in wax, the tissues were sectioned into 10 µm thin sections using a microtome. The sections were transferred to microscope slides and the paraffin wax was

removed by submerging the tissues in xylene twice for 30 minutes each. Observations of the nodule sections were collected using a standard light microscope.

Gene Expression Analysis

RNA was isolated from the tissues stored in RNAlater using the Qiagen RNeasy MiniPrep kit. The isolated RNA was reverse transcribed into cDNA and the resulting cDNA was used for PCR. Reverse transcription reactions and PCRs were performed using a BioRad Personal Thermocycler. NAD1 and PR10 primers (Domonkos et al., 2017) and DNF2 (Wang et al., 2016) were used as NAD1 and DNF2 are associated with symbiosis and PR10 is related to plant defense response. Actin primers were used a a control for gene expression as the actin gene is constitutively expressed and should produce a consistent signal on the gel for each set of plants. The PCR product was analyzed by 0.8% agarose gel electrophoresis run at 100V for about 1 hr. Gels were prepared with 1X Tris-Acetate-EDTA (TAE) buffer and DNA SafeView Classic.

Results

Plant growth assay

Growth of the plants did not differ significantly between any of the bacterial treatments. The plants inoculated with each strain of bacterial appeared healthier than the -N control plants but there was no observable difference between the plants inoculated with the different strains. At 1 WPI, inoculated plants showed more robust growth than the -N controls, but smaller plants than in the +N group (Figure 1). Similar patterns were observed at 2 WPI with no significant difference between any of the plants with the different inoculation treatments (Figure 2). At 3 WPI, the +N control group shows significantly stronger growth than the experimental plants. The plants inoculated with *ΔpilA$_1$A$_2$A$_3$* mutants appeared to have comparable growth to those inoculated with Rm 1021 bacteria, however. The *ΔpilA$_1$A$_2$* mutants were observed to be slightly smaller (Figure 3). Despite this observation, previously collected data indicates there was no difference in biomass from the different inoculants.

The results from the 6-month long assay were similar. There was, again, no significant difference in appearance between the plants inoculated with wild-type or mutant bacteria (Figure 4). There was some yellowing and loss of leaves, but it is likely that this effect was due to aging of the plants rather than an indicator of plant health. The biomass data supported these qualitative observations as there was no statistically significant difference between any of the treatments, including the +N controls.

Histological studies

Both *ΔpilA1A2* and *ΔpilA1A2A3* mutants induced abnormal nodule structure in the host plants. The nodules induced by wild-type bacteria have a smooth, oblong shape (Figure 5A) that is not consistently observed in the plants inoculated with mutant bacteria. The nodules that developed in response to the mutant bacteria often showed an abnormal, multilobed structure (Figures 5B-C). In addition to abnormal structure, nodules of plants inoculated with mutant bacteria were also observed to have inconsistent infection patterns. After staining with X-Gluc, fully infected nodules will appear consistently blue throughout the structure (Figure 5A). This phenotype was most consistently observed in the plants inoculated with Rm 1021 bacteria. Plants inoculated with mutant bacteria had a larger number of nodules that were only partially or completely uninfected, which is indicated by blue coloration in only part of the nodule or a complete absence of the blue color (Figures 5B-C).

Infection rates of nodules showed no statistically significant difference between any of the treatments at 1 WPI. At 2 WPI, however, the percents of uninfected nodules, partially infected nodules, and fully infected nodules were all significantly different between the three treatments (Figure 7). The average number of nodules per root was also significantly different between the three treatments with the *ΔpilA1A2* mutants inducing the greatest number of nodules per root. Despite having the most nodules on average per root, about half of the nodules were entirely uninfected and the other half were only partially infected. While the *ΔpilA1A2A3* plants had fewer nodules on average, they also showed significantly fewer nodules that were fully infected than the wild-type; most of the nodules were only partially infected or completely uninfected (Figure 7).

Some nodules of plants infected with mutant bacteria also appeared to have accumulation of phenolic compounds, indicated by a brown color that is distinct from the pink color associated with healthy, nitrogen-fixing nodules (Figures 5B-C & 6).

Cross-sections of nodules confirmed observations of patchy infection patterns and abnormal structure. Nodules from mutant bacteria showed cells throughout the center of the nodule that appeared uninfected; these cells are lighter in color and look deflated compared the other fully infected cells (Figures 8A-B). Nodules also showed multiple areas of meristematic growth, which is associated with areas of more and smaller cells undergoing undifferentiated growth (Figure 9A-B). Normal nodules only have one meristematic region; the multiple meristems associated with the mutant bacteria is highly abnormal.

Gene expression analysis

In RT-PCR analysis of root nodules inoculated with Rm 1021 or one of the *pilA* deletion mutants, no significant difference was observed in the expression of *PR10*, *NAD1* or *DNF2* at 1 or 2 WPI. At 3 WPI, no difference was observed in the expression of *PR10* or *NAD1*. However, *DNF2* was downregulated in root tissues of plants inoculated with $\Delta pilA_1A_2$ and $\Delta pilA_1A_2A_3$ compared to the wild-type (Fig. 10). *DNF2* is not expressed in the uninoculated control roots.

Discussion

Overall plant growth was evaluated and quantified via biomass assay. Plants inoculated with *pilA* deletion mutants were expected to be less healthy and have lower average biomass because mutant bacteria were observed to induce nitrogen fixing nodules less consistently in previous studies. The lack of nitrogen fixing activity in the nodules was expected to manifest in physical signs of stress from the plants, such as chlorosis in leaves or decreased biomass on average. However, plants did not appear to have any significant difference in appearance or growth between any of the bacterial treatments at early stages of growth (1-3 WPI). Plants were grown over a longer period of approximately 6 months to determine whether nitrogen stress symptoms would manifest in later stages of development, but plants remained similar in appearance. The biomass of each whole plant was collected and a one-way ANOVA was performed on the average whole-plant biomasses from each experimental and control group. No statistically significant difference was observed between the average biomasses of the experimental groups or the +N control at 4 WPI or at 6 MPI.

Despite the similarities in overall plant growth and appearance, mutant bacteria induced infected patterns and nodule structures that were distinct from the normal infection and development associated with the wild-type bacteria. The infection rates of $\Delta pilA_1A_2$ mutants were especially interesting because these bacteria caused the plant to produce significantly more nodules on average than were produced after inoculation with the wild-type or the $\Delta pilA_1A_2A_3$ mutants. This phenotype of higher average number of nodules per root is often observed when nodules are not effectively fixing nitrogen (Caetano-Anolles, 1990). The nitrogen-stressed plant will continue producing nodule structures in an effort to be colonized by bacteria that will fix nitrogen effectively. When the plant has enough nitrogen, the production of nodule structures is

terminated, but for as long as the plant remains stressed, it will continue to develop more nodules. The higher number of nodules per root is an indicator that nodules are not effectively fixing nitrogen (Caetano-Anolles, 1990). When the infection rates are taken into consideration, it is likely that the plant is producing more nodules to accommodate for the inability of the *ΔpilA$_1$A$_2$* bacteria to infect the nodule of the host plant consistently and fully.

It is unexpected, however, that the same phenotype of higher average number of nodules per root is not observed when plants are inoculated with the *ΔpilA$_1$A$_2$A$_3$* mutants. Our current hypothesis is that this mutant is inducing more nodules that exhibit the multilobed structure as opposed to separate nodules along the length of the root. When nodules were observed to have this structure, they were counted as a single nodule although some may have been multiple nodules that grew stacked or conjoined. More studies must be performed to determine whether the *ΔpilA$_1$A$_2$A$_3$* inoculated plants more often exhibited this stacked, conjoined, or multilobed structure than the *ΔpilA$_1$A$_2$*.

Both strains of mutant bacteria induced abnormal nodule development and structure that included the multilobed and stacked nodules. The nodules of the plants inoculated with mutant bacteria more often exhibited the multilobed structure that is a result of multiple areas of meristematic growth, which are identifiable by highly concentrated areas of cell divisions at the tips of the nodules. It is hypothesized that the pili protein is necessary for infection, as previous research has indicated that the *pilA$_2$* gene is active even as the bacteria are traveling through the infection thread. The absence of this gene may be involved in the development of this multilobed structure because the inability of the mutant to consistently infect the plants. The nodule tissue cross-sections also provided additional evidence of inconsistent infection patterns.

The nodule tissues also appeared to exhibit the abnormal, brown coloration that is indicative of phenolic compound accumulation. The accumulation of phenolic compounds is associated with defense responses from the plant and would be evidence that the mutant bacteria are inducing a defense response in the plant (Bhattacharya & Citovsky, 2010). Since it is likely that the absence of the pili protein is influencing the bacterial ability to infect the host plant, it is also likely that this reduced ability to infect it causing a defensive response from the plant. The plant may be rejecting the bacteria because they cannot effectively colonize the nodules and fix nitrogen. Future directions involve staining the nodule cross-sections to confirm that the brown color of the nodules is due to phenolic compound production from the plant.

Because it is hypothesized that the mutant bacteria are inducing a defense response from the plant, the expression of defense related genes was also evaluated. The expression of three genes related to plant defense and the Rhizobium legume symbiosis. *PR10* is a gene that is broadly related to plant defense but it appeared to be consistently expressed in all the tissues from uninoculated and inoculated plants (Bahramnejad, 2010). This result was unexpected; it was hypothesized that the *PR10* transcript would be more highly expressed in the plants inoculated with mutant bacteria because the appearance of phenolic compounds suggests that the mutant bacteria are inducing a defensive response from the plants and the expression of *PR10* would be involved in that response.

Other genes that were evaluated were *NAD1* and *DNF2*. Both genes are involved in symbiosis and necessary for the development and maintenance of nodules. The downregulation of both genes has been previously reported to result in nodule phenotypes similar to those that have been observed in this study, namely, inconsistent infection throughout the nodule and abnormally shaped nodules (Berrabah et al., 2014) (Wang et al., 2016). There appeared to be no

difference in the expression of *NAD1*, however, the *DNF2* transcript had significantly less signal on the gel for plants inoculated with the mutant bacteria compared to plants inoculated with the wild-type. In addition to being involved with nodulation, the *DNF2* gene is also involved in suppressing the immune response of the plant during symbiosis (Berrabah et al., 2015). It is hypothesized that the downregulation of *DNF2* in plants inoculated with the mutant bacteria may result in the restoration of an immune response in these plants and could explain the accumulation of phenolic compounds. *DNF2* has also been reported as necessary for maintenance of nodules. It is unclear, however, why there is only a difference in *DNF2* expression because studies on *DNF2* have reported that downregulation in this gene is also associated with upregulation of *PR10* (Berrabah et al., 2014).

In conclusion, this study provides evidence for the necessity of *pilA* genes for the full nodule infection and the normal development of nodules. Bacteria with deletions in these genes have patchy infection patterns and induce abnormal nodulation. It appears that nodules colonized by *pilA* deletion mutants also exhibit phenolic compound production and accumulation. This result, in addition to the observed downregulation of *DNF2* transcripts, suggests that the *pilA* deletion mutants are inducing a defense response in their hosts. Future directions include staining nodule cross-sections for phenolic compounds to confirm the presence of phenolics and evaluating the expression of other defense related genes.

Figures

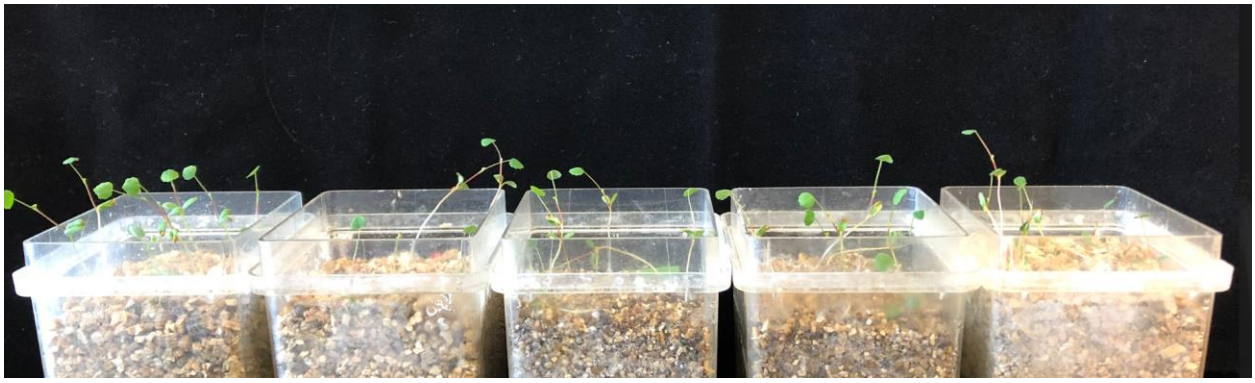


Figure 1: Plants one week post inoculation (1 WPI). Plants are ordered from right to left is as follows: +N control, -N control, Rm 1021 (wild-type), *ΔpilA$_1$A$_2$, ΔpilA$_1$A$_2$A$_3$*. No difference in appearance of any of the plants was evident.

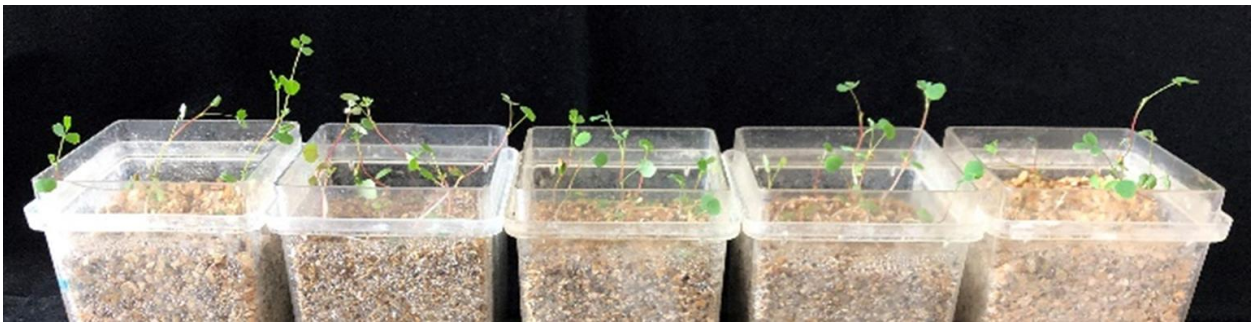


Figure 2: Plants 2 weeks post inoculation. Order of plants from right to left is as follows: +N control, -N control, Rm 1021 (wild-type), *ΔpilA*$_1$*A*$_2$, *ΔpilA*$_1$*A*$_2$*A*$_3$. No difference in appearance of any of the plants was evident.

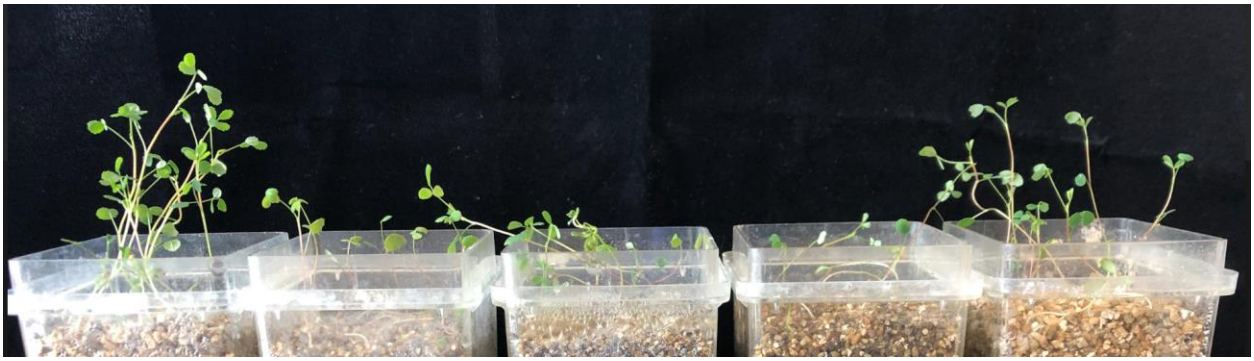


Figure 3: Plants 3 weeks post inoculation. Order of plants from right to left is as follows: +N control, -N control, Rm 1021 (wild-type), *ΔpilA$_1$A$_2$, ΔpilA$_1$A$_2$A$_3$*. +N control plants are taller and bushier than the rest of the plants, but no significant difference in the appearance of the of the plants inoculated with wild-type or mutant bacteria was evident.

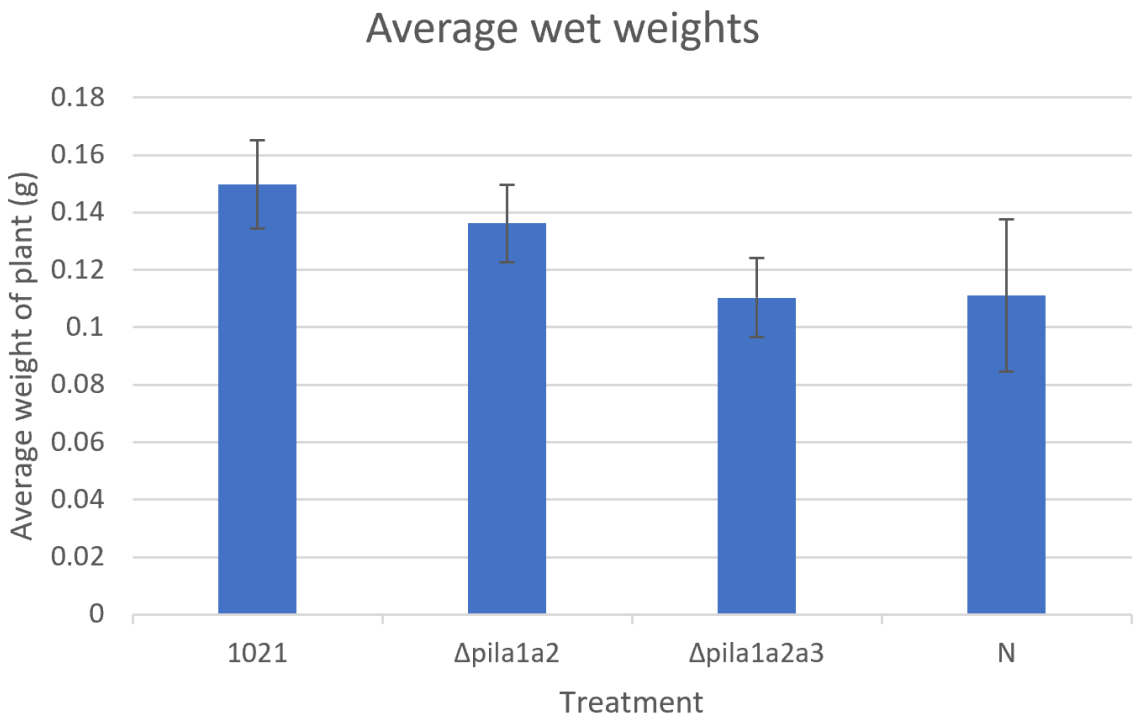


Figure 4: Average biomass of plants inoculated with Rm 1021 (WT), *ΔpilA$_1$A$_2$,* and *ΔpilA$_1$A$_2$A$_3$ S. meliloti* and +N control plants. One-way ANOVA was performed between all treatments and no statistically significant difference was observed between the data ($p = 0.2$).

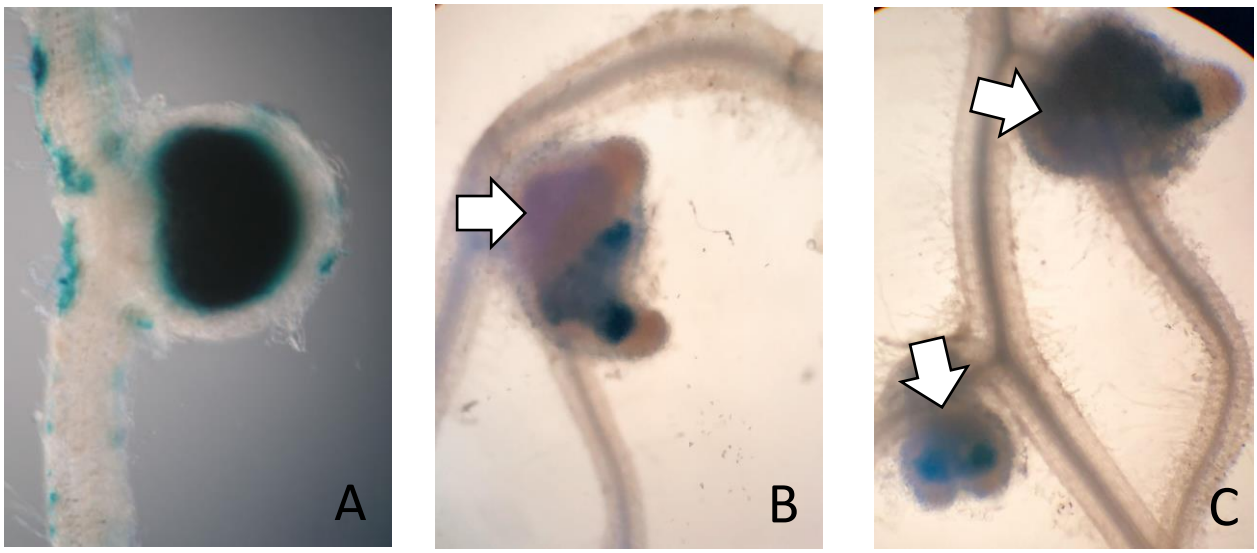


Figure 5: Localization of bacteria by GUS staining on *M. alba* root nodules 2 WPI. The blue coloration indicates the presence of *S. meliloti*. A – Inoculation with Rm 1021 (WT). Nodule exhibits normal infection pattern. B and C – Inoculation with $\Delta pilA_1A_2A_3$. Multi-lobed structure and patchy infection pattern are evident. The darker coloration at the base of the nodules may be indicative of the production of phenolic compounds as a defense response to the mutant bacteria (indicated by arrows).

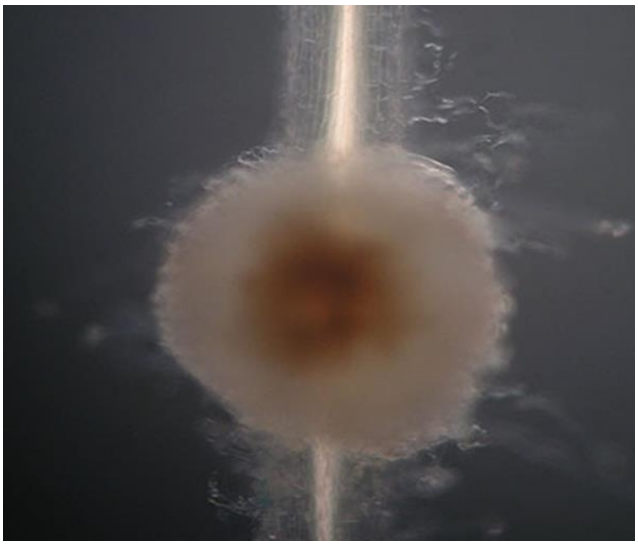


Figure 6: Root nodule inoculated with *ΔpilA$_1$A$_2$A$_3$ S. meliloti* showing brown coloration associated with phenolic compound accumulation.

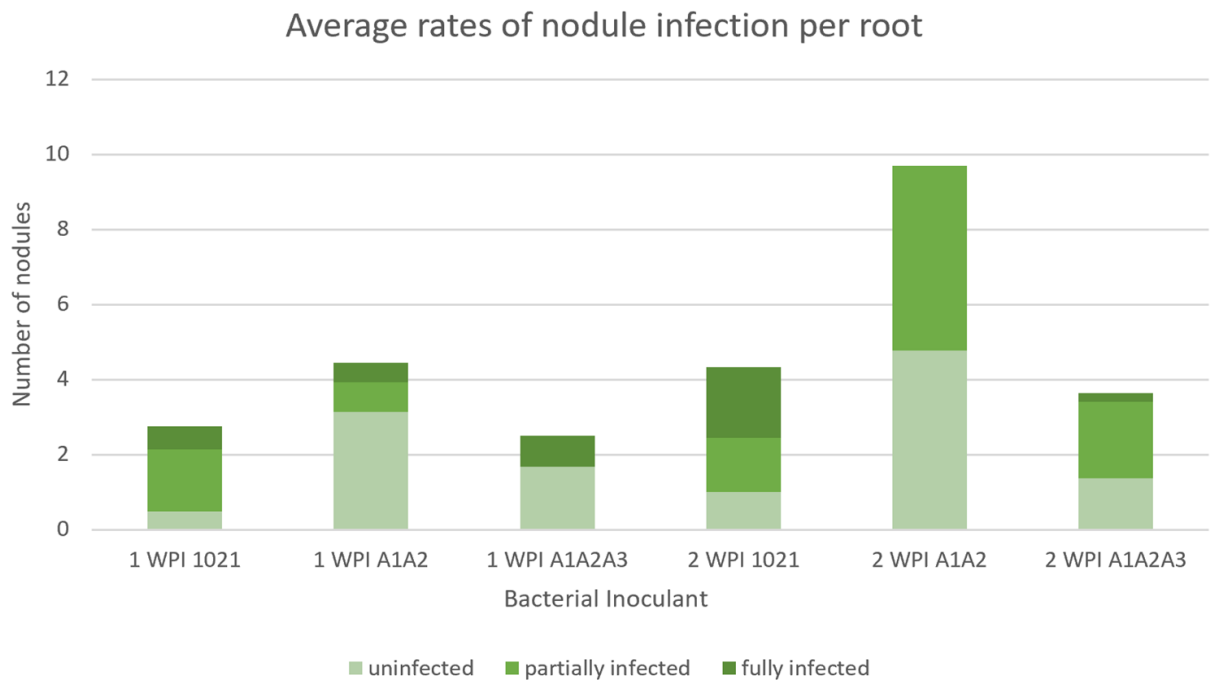


Figure 7: Graph showing infection rate data. Total bars are the average numbers of nodules per root; light green indicates uninfected nodules, medium green for partially infected nodules, and dark green for fully infected nodules. One-way ANOVA was performed for the 1 WPI and 2 WPI sets of data. No statistically significant difference was observed at 1 WPI. Average numbers of uninfected, partially infected, and fully infected nodules were significantly different between each bacterial inoculant (uninoculated, $p = 0.000135$; partially infected, $p = 0.002268$; fully infected, $p = 0.000112$).

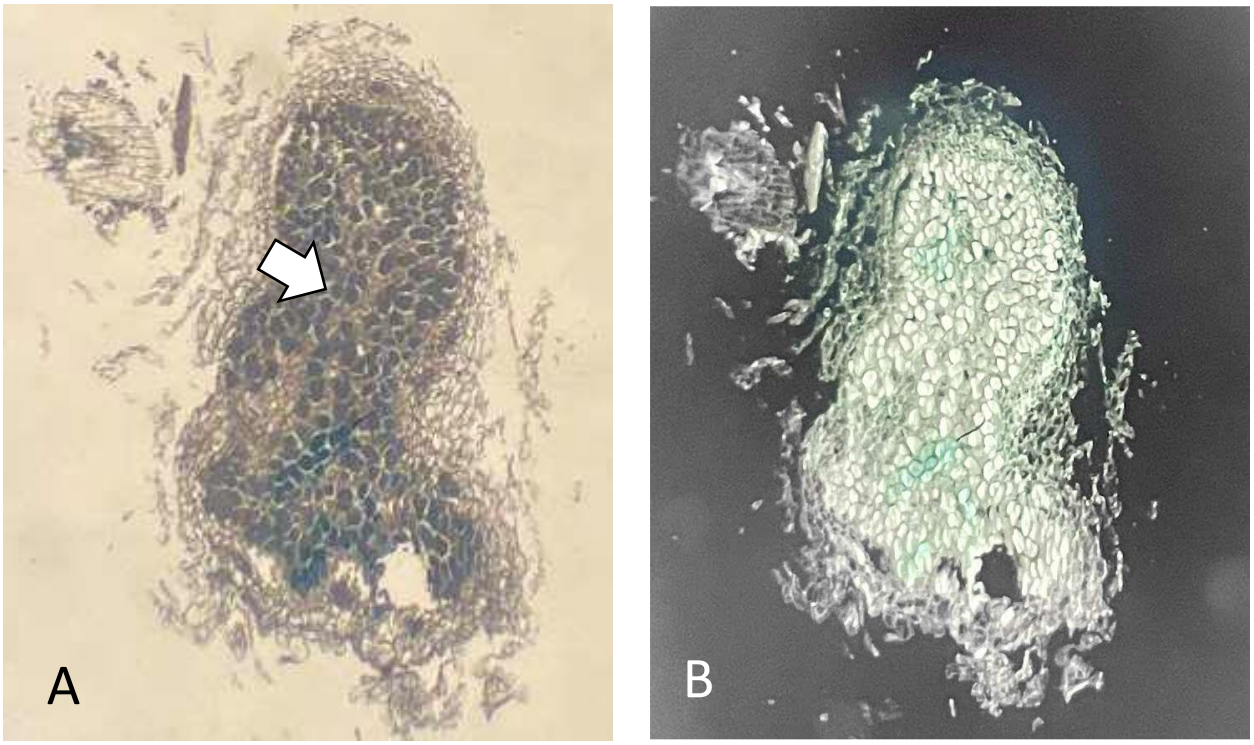
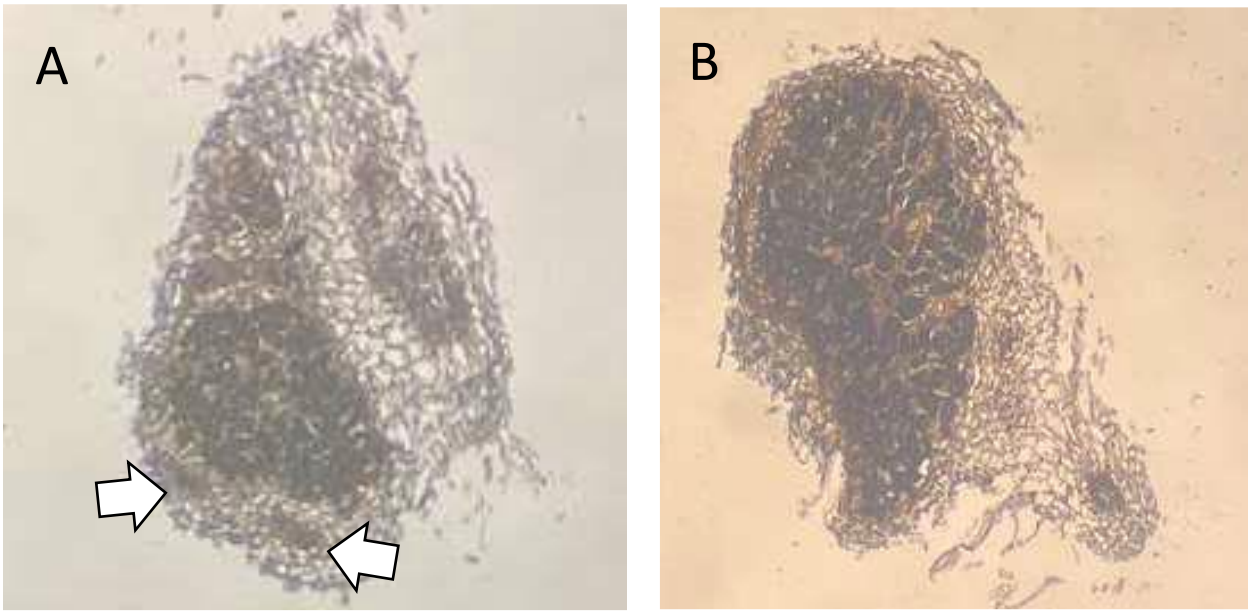


Figure 8: *ΔpilA*$_1$*A*$_2$*A*$_3$nodule sections with abnormal development. A. Bright field image. B. Dark-field image. Nodule seems to have areas of collapsed cells with lighter coloration than the fully-filled darker cells (indicated by the arrow) suggesting that these areas are not fully infected.



Figures 9A-B: *ΔpilA$_1$A$_2$A$_3$* nodule sections showing multilobed nodule structure. Rightmost image also shows multiple areas of meristematic growth, as indicated by arrows.

+N -N Rm 1021 *ΔpilA$_1$A$_2$* *ΔpilA$_1$A$_2$A$_3$*

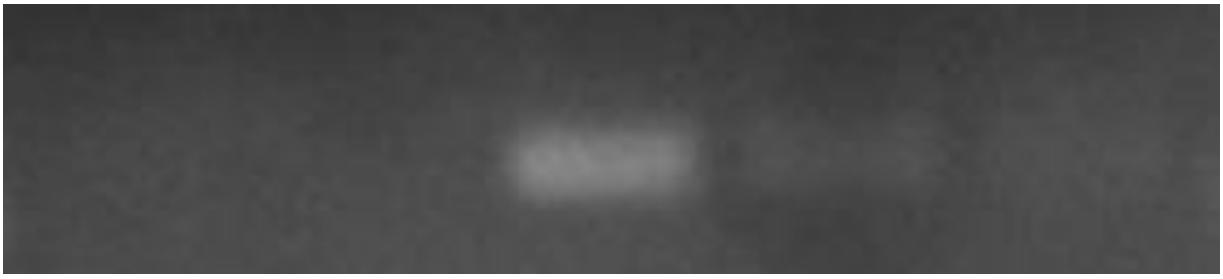


Figure 10: Gel electrophoresis of PCR product for *DNF2*. Expression of *DNF2* transcript is highly downregulated in plants inoculated with mutant bacteria. No expression is expected for +N and -N controls because expression of the gene is associated with legume-rhizobia symbiosis. PCR was performed with a constitutive actin control to ensure that difference in signal strength is due to difference in expression of the transcript.

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