

TITLE: Role of flavins in the resistance of *Sinorhizobium meliloti* –alfalfa association to *Aphanomyces* root rot.

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KEY WORDS: *Aphanomyces* root rot, *Sinorhizobium meliloti*, alfalfa association, flavins

ABSTRACT

Priority area: Novel approaches to disease and fertility management that transcend traditional organic approaches and seek to exploit and integrate biological and chemical processes.

Flavins (riboflavin, FMN and FAD) are bioactive molecules shown to have a beneficial effect on plant growth and soil quality. Recently we have found that mutations in the riboflavin biosynthetic pathway affect the ability of plant symbiont *Sinorhizobium meliloti* to secrete flavins. We also have found that flavins play a critical role in the ability of *S. meliloti* to promote plant-host adaptation to environmental stresses - the features defining the efficiency of bacterial inoculums used as a part of Rhizobium-Legume crop system. However, the role of rhizobia inoculation in the ability of the associated host-plant to resist a pathogen attack has never been evaluated. The goal of this research is to test the ability of *S. meliloti* to affect resistance/susceptibility of associated plants to *Aphanomyces* root rot and to evaluate the role of bacteria produced flavins on plant-host resistance to the pathogen.

PROJECT DESCRIPTION

One of the most severe diseases of alfalfa in temperate regions of the USA is *Aphanomyces* root rot, which is caused by the oomycete pathogen *Aphanomyces euteiches* Dreschs (Grau 1990). Symptoms of *Aphanomyces* root rot in alfalfa include stunted seedlings with chlorotic cotyledons and necrotic roots (Grau 1990). There are no fungicides that effectively control *Aphanomyces* root rot of alfalfa and recommendations for limiting losses include avoidance of fields that have poor drainage or are highly infested with the pathogen, and the use of resistant cultivars. Resistant alfalfa cultivars have better seedling vigor, higher yields and better persistence than susceptible cultivars when grown in naturally infested soils (Wiersma, Grau et al. 1995; Vincelli, Henning et al. 2000). The pathogenic interaction between *Aphanomyces* and alfalfa has been extensively studied. However, the effect of *S. meliloti* inoculation and the role of bacteria derived flavins in the resistance of the host-plant to the pathogen has not been evaluated. Our objectives are:

- To develop an assay for evaluation of alfalfa-*S. meliloti*-*Aphanomyces* interaction and
- To evaluate the role of flavins in the resistance of *S. meliloti*-alfalfa association to the fungal pathogen *Aphanomyces euteiches* by testing the ability of *S. meliloti* strains with altered ability to secrete flavins to promote host-plant resistance to the pathogen.

In this research we: (i) examined the effects of *A. euteiches* on the ability of the soil bacteria *S. meliloti* to form symbiotic association with alfalfa populations that have different levels of resistance to *Aphanomyces* root rot, (ii) evaluated the effects of inoculation of alfalfa with *S. meliloti* on the accumulation of *A. euteiches* in infected plants and the expression of disease symptoms caused by *Aphanomyces* root rot, and (iii) tested the role of rhizobia-produced flavins in the complex interaction between legume host-plant, pathogenic fungus and beneficial rhizobia.

OUTPUTS

- Work Completed:

Two types of assays for evaluation of alfalfa-*S. meliloti*-*A. euteiches* interaction have been developed. The wild type *S. meliloti* strain 1021 (Galibert, Finan et al. 2001) and its mutant carrying an in-frame deletion of riboflavin biosynthetic enzyme and exhibiting decreased flavin secretion (1021 Δ ribBA) (Yurgel, Rice et al. 2014) were used in this study. *A. euteiches* MF-1, considered the type

isolate of race 1 of the pathogen (Fitzpatrick, Brummer et al. 1998) was used for all inoculations. Two alfalfa populations were examined in this study, the variety Saranac and the germplasm WAPH 1 (Grau 1990), which are respectively the susceptible and resistant check populations used in standard tests for evaluating resistance in alfalfa to race 1 of *A. euteiches* (Fitzpatrick, Brummer et al. 1998).

Type one assay was performed in greenhouse as described previously (Vandemark, Ariss et al. 2010) with some modifications. Seed of both alfalfa populations, Saranac and WAPH-1 were planted in rows in plastic greenhouse flats containing perlite with each flat having three rows of each entry. Two weeks after inoculation with *S. meliloti* ($\sim 10^7$ CFU of bacteria per plant) between 300 and 500 *Aphanomyces* zoospores per plant were introduced to the flats. The plants which were not associated with *S. meliloti* were grown in the presence of urea and were inoculated with *A. euteiches* two weeks after planting. All flats, including control flats that were not inoculated with *A. euteiches*, were flooded for five days. After five days, all flats were allowed to drain and the experiment continued. Three weeks after inoculation with *A. euteiches*, the plants were harvested, and development of *S. meliloti* nodulation and/or *A. euteiches* infection was evaluated by q-PCR (Vandemark G.J., Ariss et al. 2010). Disease reactions of seedlings were determined 21 days after inoculation with *A. euteiches* by removing the plants from the flats and scoring 10 plants from each row for a disease severity index (DSI) ranging from 1 to 5 as follows: 1 = no necrosis of roots and hypocotyls; 2 = slight necrosis of roots and hypocotyls; 3 = necrosis of roots and lower hypocotyl, slight chlorosis of cotyledons, and moderate stunting of stem, 4 = extensive necrosis of roots, hypocotyls and cotyledons, and severe stunting of stem, and 5 = dead plant (Fitzpatrick, Brummer et al. 1998).

The second type of assay was performed in growth-chamber conditions in sterile closed Magenta boxes in sand containing N-free nutrient solution (Yurgel, Berrocal et al. 2007). 24-48 hour-old seedlings were inoculated with $\sim 10^7$ CFU of bacteria and/or ~ 300 *A. euteiches* zoospores per plant. The seedlings, which were only inoculated with fungus, were grown in the presence of 3 mM urea as a nitrogen source. Plants were harvested after 30 days post 1021 inoculation for analysis of aerial mass and harvesting of DNA from roots for Q-PCR (Vandemark, Ariss et al. 2010).

The goal of the second type of the assay was to evaluate the pathogenic interaction between standard check alfalfa populations and *A. euteiches* in small-scale plant tests performed in sterile/controlled conditions in a growth-chamber and compare the results with that obtained in the greenhouse/non-sterile condition. If the results obtained in the growth-chamber and in the greenhouse tests are comparable, the growth-chamber conditions could be used for sterile and more controlled experiments to study the response of *S. meliloti*-Medicago association to *A. euteiches* infection. Additionally, the Magenta boxes with sand represented conditions which were more beneficial for establishment of *S. meliloti* symbiosis than for development of *A. euteiches* infection, while the greenhouse experiments were designed to promote *Aphanomyces* root rot. Consequently, the incorporation of two different conditions into research could bring an additional dimension in the understanding of the plant/rhizobia/pathogens interaction.

Effect of *A. euteiches* infection on the development of *S. meliloti*-alfalfa association:

The greenhouse experiments showed that there were no significant differences between Saranac and WAPH-1 for the amount of *S. meliloti* 1021 DNA or 1021 Δ *ribBA* DNA that could be detected in the bulks from healthy, uninoculated plants (means separations across columns, Table 1). This suggests that *S. meliloti* could similarly colonize roots of both populations in the absence of *Aphanomyces* root rot.

In greenhouse experiments there were significant differences between Saranac and WAPH-1 for the amount of 1021 DNA or 1021 Δ *ribBA* DNA that could be detected in the bulks from diseased plants. In all of these cases the amount of *S. meliloti* DNA detected in the resistant germplasm WAPH-1 was

significantly greater than the amount detected in the susceptible cultivar Saranac (means separations across columns, Table 1). This suggests that in the presence of disease, *S. meliloti* was able to colonize roots of resistant plants better than susceptible plants, which suggests that having resistance is beneficial to the alfalfa–*S. meliloti* symbiosis when disease is present.

Table 1. Comparison of means^a between bulked plant samples of alfalfa standard check populations^b for quantity (ng) of *S. meliloti* DNA^c (strains 1021 and 1021 Δ ribBA), greenhouse tests. Bulks^d were collected from un-inoculated, healthy plants of both entries and also from plants inoculated with *A. euteiches* (Aph+).

Experiment 1				
	<i>S. meliloti</i> 1021 DNA (ng)		<i>S. meliloti</i> 1021 Δ ribBA DNA (ng)	
Entry	Healthy	Aph+	Healthy	Aph+
Saranac	13.50 aA	5.72 aB	14.06 aA	4.76 aB
WAPH-1	9.62 aA	9.88 bA	12.73 aA	10.63 bA
Experiment 2				
	<i>S. meliloti</i> 1021DNA (ng)		<i>S. meliloti</i> 1021 Δ ribBA DNA (ng)	
Entry	Healthy	Aph+	Healthy	Aph+
Saranac	20.91 aA	5.08 aB	14.14 aA	3.52 aB
WAPH-1	18.78 aA	12.76 bB	17.38 aA	11.19 bB

^aMeans within a column followed by the same lower case letter are not significantly different ($\alpha = 0.05$). Means within a row followed by the same upper case letter are not significantly different for ng DNA of *R. meliloti* 1021 or *R. meliloti* 1021 Δ ribBA .

^bSaranac = susceptible to *A. euteiches*; WAPH-1 = resistant to *A. euteiches*.

^cDNA was extracted from roots of bulked plant samples and tested using the primer/probe set 958F-1117T-977R. Each bulk DNA sample was tested with two replicate PCR reactions.

^dEach experiment included six bulks of 10 plants each for each population.

Table 2. Comparison of means^a between bulked plant samples of alfalfa standard check populations^b for quantity (ng) of *S. meliloti* DNA^c (strain 1021), controlled/sterile growth chamber tests. Bulks^d were collected from un-inoculated, healthy plants of both entries and also from plants inoculated with *A. euteiches* (Aph+).

	<i>S. meliloti</i> 1021 DNA (ng)	
Entry	Healthy	Aph+
Saranac	9.153 aA	15.16 aA
WAPH-1	15.23 bA	17.91 aA

^aMeans within a column followed by the same lower case letter are not significantly different ($\alpha=0.05$). ^aMeans within a row followed by the same upper case letter are not significantly different for ng DNA or mass of aerial tissue ($\alpha=0.05$).

^bWAPH-1 = resistant; Saranac = susceptible.

^cDNA was extracted from roots of bulked plant samples and tested using the primer/probe set 958F-1117T-977R. Each bulk DNA sample was tested with two replicate PCR reactions.

^d Experiment 1 and 2 combined. Experiment 1 included four bulks of 6 plants each for each population. Experiment 2 included five bulks of 6 plants each for each population.

Looking at the means separations across rows (Table 1), for both greenhouse experiments and both *S. meliloti* strains the amount of *S. meliloti* DNA detected in Saranac bulks from diseased plants was significantly less than the amount detected in healthy plants. This also suggests that disease adversely impacts the *S. meliloti*–alfalfa symbiosis. A similar relationship was also observed in Experiment 2 for the resistant germplasm WAPH-1, with significantly less *S. meliloti* DNA being detected in diseased plants. This suggests that disease may adversely impact the *S. meliloti*–alfalfa symbiosis even in resistant

materials such as WAPH-1. This is not surprising since resistance to *Aphanomyces* root rot in alfalfa is quantitatively inherited as opposed to being a case of immunity.

In growth-chamber experiments no effect of *A. euteiches* infection on the amount of *S. meliloti* DNA in both alfalfa populations, Saranac and WAPH-1, was detected (means separations across rows Table 2). Moreover, no difference between Saranac and WAPH-1 for the amount of *S. meliloti* 1021 DNA could be detected in the bulks from diseased plants (means separations across column, Table 2). A possible explanation is that in growth chamber tests, the disease did not fully progress due to the disadvantage of *A. euteiches* infection in Magenta boxes, such as sand vs. perlite used for plant growth and relatively dry condition in Magenta boxes vs. flats flooded with water for 5 days.

Effect of *S. meliloti*-alfalfa association infection on the development of *A. euteiches* infection: In both greenhouse and growth-chamber experiments for all three *S. meliloti* treatments (no *S. meliloti*, 1021, 1021 Δ ribBA/greenhouse only) of each experiment the amount of *A. euteiches* DNA detected in Saranac was significantly more than that detected in WAPH-1 (means separations across columns, Table 3 and Table 4). These results agree with previous published paper (Vandemark, Barker et al. 2002). It shows that *A. euteiches* can more successfully colonize roots of susceptible plants.

In all the treatments of greenhouse experiment 1 and for two treatments (1021 and 1021 Δ ribBA) in greenhouse experiment 2 the DSI was significantly greater for Saranac than WAPH-1 (Table 3), which also demonstrates that WAPH-1 is more disease resistant than Saranac. However, in controlled/sterile growth chamber test we did not detect significant visual differences between the plants infected with *A. euteiches* or both *A. euteiches* and *S. meliloti* 1021. We also did not detect a decrease in the mass of the plants infected with only *A. euteiches* compared to the mass of plants infected with both, *A. euteiches* and *S. meliloti* 1021. This suggested that *A. euteiches* infection did not fully progress due to the disadvantage of *A. euteiches* infection in Magenta boxes.

Table 3. Comparison of means^a between bulked plant samples of alfalfa standard check populations^b for disease severity index (DSI) ratings^c and quantity (ng) of *A. euteiches* DNA^d, greenhouse tests. Bulks were collected from plants of both entries tested with three treatments: 1. Not inoculated with *S. meliloti* (*S. meliloti* -); 2. Inoculated with *S. meliloti* 1021, and 3. Inoculated with *S. meliloti* 1021 Δ ribBA.

Experiment 1						
	<i>A. euteiches</i> DNA (ng)			DSI		
Entry	<i>S. meliloti</i> -	1021	1021 Δ ribBA	<i>S. meliloti</i> -	1021	1021 Δ ribBA
Saranac	0.97 aA	0.94 aA	0.47 aB	3.93 aA	3.87 aA	3.88 aA
WAPH 1	0.17 bA	0.18 bA	0.06 bA	3.12 bA	3.08 bA	2.81 bA
Experiment 2						
	<i>A. euteiches</i> DNA (ng)			DSI		
Entry	<i>S. meliloti</i> -	1021	1021 Δ ribBA	<i>S. meliloti</i> -	1021	1021 Δ ribBA
Saranac	1.11 aA	0.47 aB	0.41 aB	4.0 aA	4.0 aA	4.0 aA
WAPH 1	0.13 bA	0.01 bB	0.01 bB	3.0 aA	2.75 bB	2.55 bB

^aMeans within a column followed by the same lower case letter are not significantly different ($\alpha = 0.05$). ^aMeans within a row followed by the same upper case letter are not significantly different for ng DNA or DSI ($\alpha = 0.05$).

^bWAPH-1 = resistant; Saranac = susceptible.

^cEach experiment included six bulks of 10 plants each for each population. Prior to bulking, plants were individually scored using a DSI scale of 1 (healthy)–4 (extensive necrosis of roots, hypocotyls and cotyledons, and severe stunting of stem).

^dDNA was extracted from roots of bulked plant samples and tested using the primer/probe set 136F-161T-211R. Each bulk DNA sample was tested with two replicate PCR reactions.

The effect of inoculating plants with *S. meliloti* on expression of Aphanomyces root rot symptoms (either DNA or DSI) was somewhat inconsistent in both greenhouse and growth-chamber conditions. However, the results do suggest that inoculation of plants with *S. meliloti* may ameliorate disease. This is especially true of the data based on real-time PCR results, which should afford better quantitative analysis than use of an integer scale for DSI. For both alfalfa populations in greenhouse experiment 2 the amount of *A. euteiches* DNA detected in plants that were not inoculated with *S. meliloti* was significantly greater than the amount detected in bulks of plants that were also inoculated with either isolate of *S. meliloti* (means separations across rows, Table 3). Additionally, in growth-chamber conditions, significantly more *A. euteiches* DNA was detected in WAPH-1 plants that were not inoculated with *S. meliloti* compared to that in the WAPH-1 plants associated with *S. meliloti* (means separations across rows, Table 4).

Table 4. Comparison of means^a between bulked plant samples of alfalfa standard check populations^b for mass^c of aerial tissue and quantity (ng) of *A. euteiches* DNA^d, controlled/sterile growth chamber tests. Bulks were collected from plants of both entries tested with two treatments: 1. Not inoculated with *S. meliloti* (*S.meliloti*-) and 2. Inoculated with *S. meliloti* 1021.

Entry	<i>A. euteiches</i> DNA (ng)	
	<i>S. meliloti</i> –	1021
Saranac	4.675 aA	3.206 aA
WAPH-1	1.075 bA	0.068 bB

^aMeans within a column followed by the same lower case letter are not significantly different ($\alpha=0.05$). ^aMeans within a row followed by the same upper case letter are not significantly different for ng DNA or mass of aerial tissue ($\alpha=0.05$).

^bWAPH-1 = resistant; Saranac = susceptible.

^cExperiment 1 and 2 combined. Experiment 1 included four bulks of 6 plants each for each population. Experiment 2 included five bulks of 6 plants for each population. DNA was extracted from roots of bulked plant samples and tested using the primer/probe set 136F-161T-211R. Each bulk sample was tested with two replicate PCR reactions.

Sinorhizobium meliloti flavin secretion and bacteria-host interaction: role of the bifunctional RibBA protein: *S. meliloti* 1021 secretes a considerable amount of riboflavin. This precursor of the cofactors flavin mononucleotide and flavin adenine dinucleotide is a bioactive molecule that has a beneficial effect on plant growth. The *ribBA* gene of *S. meliloti* codes for a putative bifunctional enzyme with dihydroxybutanone phosphate synthase and GTP II activities, catalyzing the initial steps of the riboflavin biosynthesis pathway. We showed that an in-frame deletion of *ribBA* does not cause riboflavin auxotrophy or affect the ability of *S. meliloti* to establish an effective symbiosis with the host plant but does affect the ability of the bacteria to secrete flavins, colonize host-plant roots, and compete for nodulation. A strain missing the RibBA protein retained considerable GTP cyclohydrolase II activity. Based on these results, we hypothesized that *S. meliloti* has two partly interchangeable modules for biosynthesis of riboflavin, one fulfilling the internal need for flavins in bacterial metabolism and the other producing riboflavin for secretion. Our data also indicated that bacteria-derived flavins play a role in communication between rhizobia and the legume host and that the RibBA protein is important in this communication process even though it is not essential for riboflavin biosynthesis and symbiosis. A paper describing this research has been published in MPMI (2014).

We also tested the role of rhizobia-produced flavins in the complex interaction between legume host-plant, pathogenic fungus and beneficial rhizobia. Our data do not suggest any differences between 1021 or 1021 Δ *ribBA* in their ability to ameliorate disease caused by *A. euteiches* (Table 3). Additionally, we tested the ability of 1021 Δ *ribBA* to decrease envelopment of *A. euteiches* in alfalfa in growth-chamber

conditions in several independent experiments. However, the results of these experiments were inconsistent.

- Publications, Handouts, Other Text & Web Products:

Sinorhizobium meliloti flavin secretion and bacteria-host interaction: role of the bifunctional RibBA protein. Yurgel SN, Rice J, Domreis E, Lynch J, Sa N, Qamar Z, Rajamani S, Gao M, Roje S, Bauer WD. Mol Plant Microbe Interact. 2014 May; 27(5):437-45.

Protocols published:

Assay for GTP cyclohydrolase II activity in bacterial extracts. Yurgel SN, Sa N, Rice J and Roje. 2014. www.bio-protocol.org

Quantification of flavin production by bacteria. Yurgel SN, Lynch J, Rice, Adhikari N, Roje S. 2014. www.bio-protocol.org

Abstract/Poster: *Sinorhizobium meliloti* flavin secretion and bacteria-host interaction: role of the bifunctional RibBA protein. Yurgel SN, Rice J, Lynch J, Sa N, Roje S, Bauer WD. Plant Biology 2014. Annual scientific meeting of the American Society of Plant Biologists, July 12-16, 2014, Portland.

- Outreach & Education Activities:

IMPACTS

- Short-Term: Two assays to study alfalfa/*S. meliloti*/*A. euteiche* interaction have been developed. The comparison of these assay showed that the greenhouse experiments are more reliable for detection of the effect of *A. euteiche* infection on *S. meliloti*-alfalfa association. However the sterile growth-chamber experiments could be used to study the effect of *S. meliloti* inoculation on the development of Aphanomyces root rot. Our data showed that (i) inoculation with rhizobia could improve the ability of the associated host-plant to resist a pathogen attack and (ii) *A. euteiche* infection adversely impacts the *S. meliloti*-alfalfa symbiosis. We did not detect a significant role of bacteria-derived flavins in the improved resistance of rhizobia associated alfalfa plants to *A. euteiche* infection. The manuscript describing this research is in preparation.
- Intermediate-Term: The data obtained in this research provide a foundation for comprehensive investigation of mechanisms of anti-fungal effect *Sinorhizobium* symbiosis on Medicago-*A. euteiche* association and for a search for alfalfa-*Sinorhizobium* combination with high level of symbiotic nitrogen fixation and improved resistance to fungal infection (see RECOMMENDATIONS FOR FUTURE RESEARCH).
- Long-Term: The results obtained in this research could promote the creation of rhizobial inoculums, which provide host-plant with both (i) sufficient nitrogen for growth and (ii) antifungal protection under *A. euteiche* attack.

ADDITIONAL FUNDING APPLIED FOR / SECURED

Five applications for alternative sources of competitive non-university funds have been submitted through CAHNRS Office of Research/ARC: (1) Full proposal to USDA AFRI (2013), (2-3) Pre-proposal to NSF (2013 and 2014), and (4-5) R21 proposal (2013) and R01 (2014) to NIH-NIAID.

GRADUATE STUDENTS FUNDED

None

RECOMMENDATIONS FOR FUTURE RESEARCH:

This project is far from completion. The next logical step is to evaluate the role of symbiotic bacteria in the improved resistance of Medicago to *A. euteiches* infection by dissecting the role of micro- and

macro- symbionts in the control of *A. euteiches* growth and uncovering the molecular background of the effect of a rhizobium-legume association on the development of *A. euteiches* root rot and on its regulation at a cellular level. Based on the results obtained in the BIOAg funded research we hypothesize that the presence of rhizobia can directly inhibit pathogen development on the host root or/and stimulate the local and systemic induction of defense systems in the rhizobia-associated plants by affecting the expression of pathogen defense-related (PR) genes. The approaches to test this hypothesis might include:

(i) Analysis of host-plant response to *A. euteiches* infection in the presence of *Sinorhizobium* by (i) employing RT-PCR to examine the differences in the level of expression of the genes induced by *A. euteiches* between the model legume *Medicago truncatula* plant inoculated with *Sinorhizobium* and uninoculated plants; (ii) and by deep transcriptome analysis (RNA-seq) of the response of *M. truncatula* to *A. euteiches* infection and/or *Sinorhizobium* inoculation. This approach will allow to identify a broad set of PR genes in legumes.

(ii) Analysis of how cross-communication between rhizobia and *A. euteiches* is stimulated by legumes by studying the changes in the transcriptome (RNA-seq) profiles of *Sinorhizobium* and *A. euteiches* after their exposure to each other and/or to the host-plant. These experiments will allow to identify rhizobia cell responses (genes) that control *A. euteiches* infection.

Another direction of the future research is to test the antifungal effect of rhizobia inoculation in other rhizobium/legume(pea, chickpea)/pathogen systems.

Additionally, a search for the most beneficial host-plant/rhizobia combination, which could provide the host-plant with both (i) sufficient nitrogen for growth and (ii) antifungal protection under *A. euteiche* attack, is an important direction of the research aimed to improve resistance of the legume crops to biotic stresses. Very briefly, this research could include (i) isolation of rhizobia from the nodules of widely cultivated legumes, such as alfalfa, pea or chickpea, grown in agricultural fields, (ii) and testing the ability of some rhizobial representatives to form effective nitrogen fixing symbiosis and protect host-plants from pathogen infection.

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Yurgel, S. N., J. Rice, et al. (2014). "Sinorhizobium meliloti flavin secretion and bacteria-host interaction: role of the bifunctional RibBA protein." Mol Plant Microbe Interact **27**(5): 437-445.