TITLE:

The Search for Acid-tolerant Rhizobia to Improve Pulse Production

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KEY WORDS

nitrogen fixation, rhizobia, soil acidification, acid tolerance, chickpea, lentil

ABSTRACT

Soil acidification is a growing challenge in Washington's agricultural soils due to long term application of ammonia-based fertilizers. Acidic soil is detrimental to legume-rhizobia symbioses and nitrogen fixation. We seek to find acid tolerant and effective strains of *Mesorhizobium* spp. and *Rhizobium leguminosarum*, which associate with chickpeas and dry peas/lentils, respectively. The ultimate mission of this research is to maintain or increase production and biological nitrogen fixation of three major Washington pulse crops despite unfavorable and worsening soil conditions.

Eighty putative isolates of *M. ciceri* and 103 putative *R. leguminosarum* bv. *viciae* from chickpeas and lentils growing in Palouse area soils were studied. Thirty percent of *M. ciceri* isolates and 69% of *R. leguminosarum* were moderately tolerant of pH 5.5, but only 7% and 17%, respectively, were even slightly tolerant of pH 4.5 in laboratory tests. Robust analysis of six regions of bacterial DNA, determined that 20 unique *R. leguminosarum* and 34 unique *M. ciceri* have been collected. With this information, the unique and more acid-tolerant isolates have been identified for further studies in soil conditions. The slide into acidified soil conditions in the Palouse is likely to dramatically reduce the growth and survival of most of the current rhizobial symbionts of our pulse crops.

PROJECT DESCRIPTION

Cool season pulse yields are declining the Pacific Northwest due to ongoing soil acidification due to ammoniacal fertilizer use and ion loss through leaching and harvest. Rising acidity is detrimental to crop root growth and yield, and is also detrimental to the legumes' symbionts, *Mesorhizobium ciceri* (chickpea symbiont) and *Rhizobium leguminosarum* bv. *vicae* (pea and lentil symbiont). Nitrogen fixation supplied by this symbiosis is worth tens of millions of dollars to Palouse growers annually. Loss of this service through soil acidification and lack of acid-tolerant rhizobial inoculants is becoming a very expensive problem. This project seeks to identify rhizobial strains for chickpea, pea, and lentil that grow in acidic conditions and fix nitrogen effectively.

Nearly 200 isolates of bacteria were collected from legume root nodules growing in the Palouse between 2010 and 2015. In this study, six genetic regions were sequenced to identify true *M. ciceri* and *R. leguminosarum* and to assess their genetic diversity. Isolates that appear to be genetically identical can then be represented in phenological testing by a single isolate. The growth capacity of 43 *M. ciceri* and 54 *R. leguminosarum* isolates at low pH were assessed in laboratory tests. Isolates were screened for growth at pH 6.5, 5.5, and 4.5. Growth was also assessed at different levels of pH buffering to further assess isolates for potential to perform well in acidic soils.

Most isolates had some tolerance to acidic conditions. Using a relative scale of 0-3 to represent levels of growth, 26 of 43 *M. ciceri* and 44 of 54 *R. leguminosarum* attained a growth score of at least 1.0 at pH 5.5. A score of 1 indicated survival and limited growth of the isolate, or a slight tolerance. However only 13 of 43 *M. ciceri* and 37 of 54 *R. leguminosarum* attained a growth score of at least 1.5 at pH 5.5. A score of 1.5 indicated a moderate tolerance and growth. And, only 3 *M. ciceri* and 9 *R. leguminosarum* grew to score 1.0 at pH 4.5. Few cultivated fields in the Palouse still have pH as high as 5.5, with most between 4.5 and 5.5. In further trials with the more acid tolerant isolates, differing levels of buffering have been used in the bacterial growth media. A high level of buffering means that pH is more resistant to change due to a higher concentration of buffering ions that maintain the initial pH. As has been seen in prior studies, calcium is required in solution for the bacteria to grow in acidic conditions.

Our most interesting data thus far indicate that 1) isolates that grow in acidic media actually change the pH of their environment, increasing pH to near neutral, 2) this ability to withstand and grow at a low pH requires Ca (also observed in previous studies), 3) level of buffering matters, with highly buffered acidic media supporting less growth than weakly buffered acidic media, 4) a few isolates can grow in highly buffered pH 5.5 media but measurable growth begins after several weeks rather than in 2-4 days at pH 6.5.

In addition to testing acid tolerant isolates in an array of chemical conditions, genetic studies were undertaken. An initial sequencing of the 16S rDNA verified the isolates that were of the expected species. These isolates were re-purified and further characterized by sequencing *atpD*, *GSI*, *GSII*, *nodC*, and *recA* genes. Among the *R. leguminosarum* isolates, only 20 were found to be genetically unique using this robust 5-gene analysis. Among the *M.* spp., 34 isolates were unique. In both groups, the replicate isolates were primarily found among isolates from the same farm. However in the *R. leguminosarum* group we also found 18 identical isolates from the Oaksdale, WA, and Colfax, WA, areas.

Greenhouse trials were also initiated but further genetic information was important to obtain before completing these trials. Further greenhouse and field trials will be needed to assess nitrogen fixation in acidic soils. Improved commercial products may be possible using isolates that fix nitrogen well in acidic soil conditions, or using amendments that provide nutritional support for improved acid tolerance.

OUTPUTS

Work Completed:

- The bacterial isolates used in this study have been collected from multiple commercial and research fields in the Palouse region over the past 7 years. All were isolated directly from the nodules of chickpea, lentils, or peas. To confirm their identity as *R. leguminosarum* or *M. ciceri*, four genetic regions (16S rDNA, *GSII, recA*, and *nodC*) were sequenced for 103 putative rhizobia isolates and 80 putative mesorhizobia. Through BLAST analysis of these sequences, seventy-one isolates were confirmed as *M. ciceri*, an additional 3 as other *M. spp*., and 89 as *R. leguminosarum*. To improve the assessment, *GSI* and *atpD* genes were also sequenced. Phylogenetic trees were developed using these sequences to identify unique and duplicate isolates. A subset of unique isolates has been identified for further testing.
- A defined and buffered acid growth media was developed and used to optimize an *in vitro* screen for acid tolerance in rhizobia. Fourty-three isolates of *M ciceri* and 54 isolates of *R. leguminosarum* have been evaluated for growth potential in acidic conditions (Figure 1).
- Selected isolates with some tolerance to acidic conditions were grown for longer periods in broth media using a range of chemical conditions. The ultimate goal is to identify isolates that can support good nitrogen fixation in acidic soils. Soils with low pH also often have high aluminum, low calcium, and/or low available phosphorus concentrations. Since these factors can in themselves restrict rhizobial growth and nitrogen fixation, it is important to understand their different effects on rhizobia. Trial conditions have included pH 6.5, 5.5 and 5.0 at high or low calcium, aluminum, and phosphorus conditions. Isolates that grew at pH 5.5 buffered solution required high Ca (Figure 2). Continued growth trials are assessing the range of aluminum tolerance and buffering tolerance.
- Greenhouse trials to assess nitrogen fixation effectiveness were initiated.

PUBLICATIONS, OTHER PRODUCTS:

Piaskowski, J., G. Vandemark, N. Pierre-Pierre, L. Carpenter-Boggs. Diversity of *Mesorhizobium ciceri* and *Rhizobium leguminosarum* in soils of the Palouse region. In preparation.

OUTREACH AND EDUCATIONAL ACTIVITIES:

- Carpenter-Boggs, L., and J. Piaskowski. The Acid Test: Searching for Acid Tolerant Rhizobia. Presentation to grants board, US Dry Pea and Lentil Council.

- Undergraduate researcher Jessica Puente Arroyo has been engaged in this research. She has learned culture storage and maintenance, and performed the sequencing PCR for genetic analysis of these cultures.

IMPACTS

- Short-Term:

The acid tolerance information on these isolates is useful information for characterizing the rhizobial collection held jointly by WSU and the USDA-ARS. These data have been matched with DNA sequence information on the isolates for use by other researchers. A genetically and functionally diverse array of isolates will be entered to the National Rhizobial Collection for use by other researchers. The information generated by this line of study will expand knowledge about the strains, conditions, and nutritional supplements that can improve survival of rhizobia and nitrogen fixation in acidic soils.

- Intermediate-Term:

With further laboratory, greenhouse, and field testing, new inoculants and amendments are foreseen that support and increase nitrogen fixation in acidic soils. The value of nitrogen fixation per year can be $20 - $60 per acre in conventionally fertilized fields or over $500 per acre in organic production. Use of nitrogen fixing crops in rotation also reduces the use of additional nitrogen fertilizers which have a worse acidifying effect.

- Long-Term:

This study is part of a longer term effort to characterize existing populations of rhizobial bacteria that associate with legumes grown in the Palouse, and to improve inoculants and amendments for conditions found in soils of the PNW. The long term effect will be maintained or improved diversity of cropping rotations including chickpeas, peas, and lentils; improved nitrogen fixation in acidic soils, and improved environmental and economic sustainability.

ADDITIONAL FUNDING APPLIED FOR / SECURED

Additional funds have been requested from the US Dry Pea and Lentil Council and the USDA-ARS Postdoctoral Research Associate Program. We are also preparing for application to the NIFA Foundational Program in 2017 (Plant Health and Production and Products).

GRADUATE STUDENTS FUNDED

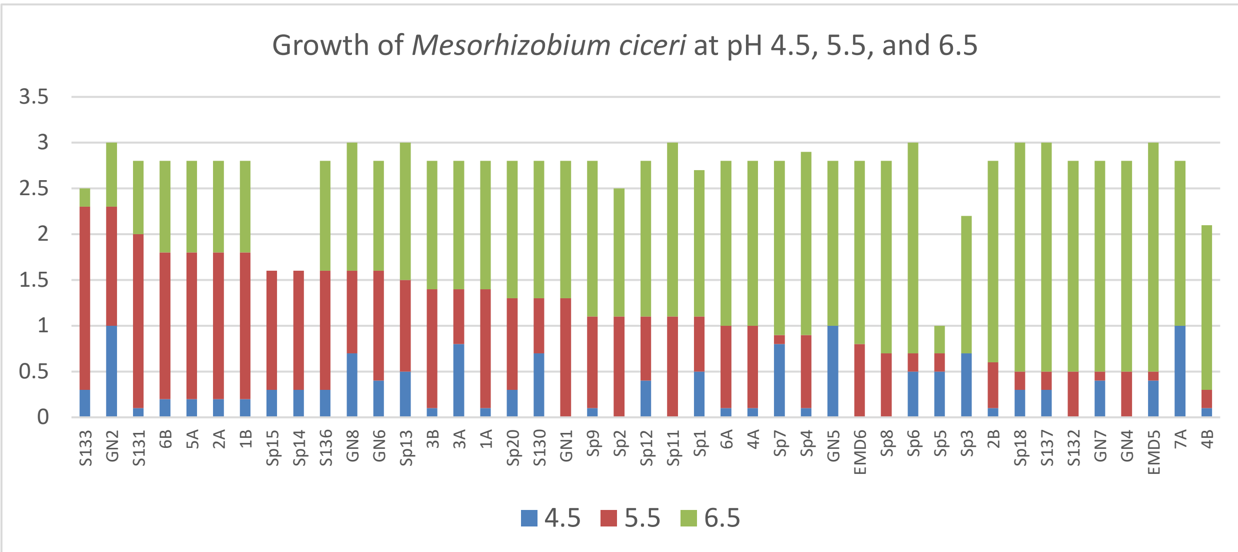
Catherine Crosby, PhD Soil Science.

RECOMMENDATIONS FOR FUTURE RESEARCH

Extensive efforts should be made to collect rhizobial bacteria in agricultural and natural environments, and to characterize their genetic and phenological diversity. Testing is needed to assess the growth and nitrogen fixation effectiveness in a wide variety of laboratory and soil conditions. The mechanisms of rhizobial tolerance to acidic solutions and soils should be determined in order to develop and optimize new inoculants and amendments. Field trials are needed to complement the results from the *in vitro* trials.

Figure 1. Growth of *Mesorhizobium ciceri* (a) and *Rhizobium leguminosarum* (b) isolates in laboratory tests at pH 4.5 (blue), 5.5 (red), and 6.5 (green).

a.



b.

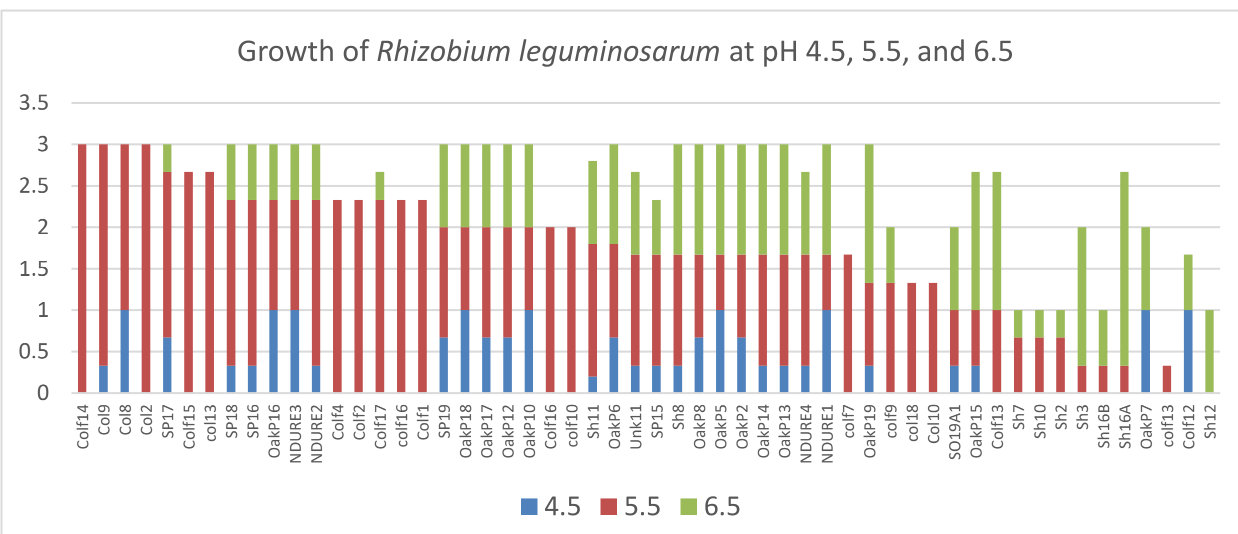


Figure 2. *M. ciceri* grown at pH 6.5 and pH 5.5 with increased Ca and buffer concentration of 5g/L succinate. Strain S136 M-1 was able to overcome the pH 5.5 challenge in 8 days, while the other strains required at least 15 days to reach exponential phase. At pH 6.5 isolates reached exponential phase in 4 days. All strains that grew at pH 5.5 with increased Ca raised the pH of the growth environment.