EVALUATIONS OF DIFFERENTIAL ACQUISITION AND TRANSMISSION OF BNYVV BY *POLYMYXA BETAE* ISOLATES FROM THROUGHOUT THE UNITED STATES

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Background:

Polymyxa betae is the vector of several soil-borne viruses of sugarbeet (Abe and Tamada 1986, Liu and Lewellen 2008, Wisler et al. 1994, Kaufmann et al. 1992), including *Beet necrotic yellow vein virus* (BNYVV), the cause of rhizomania (Tamada and Baba 1973). BNYVV is transmitted by zoospores of this fungus-related microorganism that swim in water films and infect feeder roots of sugarbeet plants. *P. betae* is an obligate parasite and is found in virtually every soil in which sugarbeet is grown (Liu and Lewellen 2007). The pathogen forms long-lived resting spores clustered together to form cystosori (Fig. 1) that can survive in the field for many decades. The life cycle and mode by which *P. betae* infects sugarbeet roots are well documented (Keskin 1964). *P. betae* is usually not considered to cause measurable damage to sugar beet, however, in controlled tests, it has been shown to cause reductions in yield (Wisler et al 2003, Liu and Lewellen 2008), and studies have shown that nearly half the protein changes identified in sugarbeet associated with BNYVV infection were induced by the presence of *P. betae* in roots (Larson et al., 2008).

Initial studies are examining different pure culture *P. betae* isolates from several sugarbeet production regions, including several from Red River Valley region. In earlier research at Salinas by H.-Y. Liu, methods developed by Kingsnorth et al. (2003) for quantification of *P. betae* in infected tissue by real time PCR were modified to screen *Beta* germplasm for possible resistance to *P. betae* (H-Y. Liu, unpublished). The results of the real-time PCR quantification assay for *P. betae* ranged from 9 to 881,000 copies of the target sequence, with the lower values reflecting resistance to colonization by the fungal vector. *Beta patelaris*, *Beta procumbens*, and *Beta webbiana* were highly resistant to *P. betae* with an average of 52 copies of the target sequence; this is in agreement with previous findings (Paul et al. 1992, 1993). The four commercial hybrids ranged from 48,000 to 881,000 copies with the variety 'Angelina,' a variety highly resistant to BNYVV, being most susceptible to *P. betae* of those examined. This result was supported by microscopic examinations showing that 'Angelina' had the most *P. betae* cystosori (clusters of long-term resting spores) embedded in the roots. The remaining sugarbeet lines had a similar range of susceptibility with the exception of 3 breeding lines that had low levels of *P. betae* colonization. A separate proposal focusing on characterization and development of potential resistance to *P. betae* was submitted to the Beet Sugar Development Foundation and is distinct but complementary to this proposal.

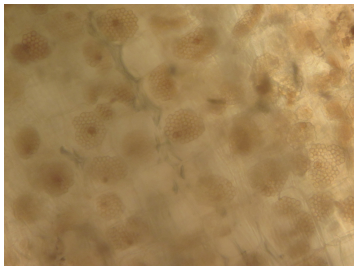

Figure 1. Light microscopy of *P. betae* cystosori visualized in sugarbeet roots. Cystosori are the clusters of spherical particles shown in root cells below. In this photo, there are cystosori in most of the cells on the surface of the root.

Objectives:

1. Examine differential acquisition of BNYVV by *P. betae* isolates collected from several US sugarbeet production regions.

2. Examine differential transmission of BNYVV by *P. betae* isolates collected from several US sugarbeet production regions.

3. Begin studies examining acquisition and transmission of BNYVV isolates varying in disease severity to determine if severity relates to vector efficiency of *P. betae*.

Summary of Project to Date:
Methods for quantification of BNYVV levels using real time (quantitative) RT-PCR were developed previously and clearly differentiate accumulation of not only BNYVV but also two other soil-borne sugar beet-infecting viruses; *Beet soil-borne mosaic virus* (BSBMV) and *Beet soil-borne virus* (BSBV) using differential probes. This was described in the 2012 Annual Report for a previous project. Previously published methods for quantification of *P. betae* were evaluated by the Martin Lab (collaborator), but did not have sufficient specificity. As a result new methods were developed over the past year by the Martin Lab for quantification of *P. betae*. Validation studies completed in November 2014 demonstrated the current probes are effective, and they are now being used in analysis of test material along with the BNYVV probes for quantification of *P. betae* and BNYVV levels in sugarbeet and wild beet.

Pure culture isolates of *P. betae* collected from sugarbeet production regions around the U.S. have been isolated and increased over the past year and a half (see 2013 report). Studies are in progress to evaluate differential acquisition and transmission of BNYVV by these isolates.

Acquisition of BNYVV by P. betae isolates collected from several US sugarbeet production regions.

Single cystosorus isolates of Polymyxa betae were isolated and increased during the first year and a half of this project from most of the major sugarbeet production regions in the United States, including Minnesota, Idaho, Colorado, Nebraska, Wyoming, Oregon, and California. This was to obtain maximum potential variability among *P. betae* isolates from throughout the US. Permits from USDA-APHIS are in place for working with these cultures from throughout the US.

Once each pure culture *P. betae* isolate has been sufficiently increased and *P. betae* levels determined, wild beet (*Beta macrocarpa*) is planted into each soil containing a different pure isolate of *P. betae*. *B. macrocarpa* plants are inoculated at the two leaf-stage with a single local lesion (pure culture) isolate of BNYVV pathotype A, the traditional form of BNYVV common in the US. *B. macrocarpa* was selected for these experiments because BNYVV infection is more uniform and moves systemically throughout the plant more efficiently than in sugarbeet. This allows BNYVV to move readily into root tissues where *P. betae* is active. Some pots of each *P. betae* isolate containing *B. macrocarpa* plants remain uninoculated as controls for each *P. betae* isolate. Co-cultivation of virus and vector is maintained for a period of 6 weeks, with sampling of roots weekly beginning at 2 weeks post-virus inoculation, continuing weekly.

At each collection time point, seedling roots were carefully washed and the entire root system was homogenized and ground to a fine powder with liquid nitrogen and stored at -80C until processing. DNA and RNA are prepared separately using established methods from each root and/or cystosori sample (same root source used for extraction of both RNA and DNA) for use in determining *P. betae* and BNYVV levels, respectively. The RNA and DNA extracts were then assessed for purity and integrity using NanoDrop spectrophotometry (Thermo Scientific, Delaware, USA) and agarose gel electrophoresis. Quantitative RT-PCR was performed on RNA for measuring BNYVV titers, and quantitative PCR was performed on DNA for measuring *P. betae* levels. Results compare the amount of *P. betae* present to the amount of BNYVV present in the *P. betae*. Experiments are being conducted in sequential replicated experiments. Not all isolates can be compared simultaneously due to limits on the number of samples that can fit into individual growth chambers. However, experimental controls allow comparison across experiments based on standards used as controls, and allow for comparison of PCR efficiency.

Although current results are preliminary, we are observing a parallel between the amount of *P. betae* present in roots of *Beta macrocarpa* plants used in virus acquisition tests (data not shown), and the amount of virus in these samples tested to date (Fig. 2); however, we have only just begun evaluating different *P. betae* isolates in comparison to one another, and additional replications will be needed to determine if initial results will prove to be consistent. So far we have not observed dramatic differences in levels of virus accumulating in different isolates of *P. betae*; however,we have only evaluated a small number of isolates so far. This is very preliminary information.

Studies evaluating transmission will be conducted once we complete analysis of acquisition, since the acquisition studies are the method used to introduce BNYVV into each pure culture *P. betae* isolate. These studies will evaluate

variation in transmission of BNYVV to sugarbeet using the same *P. betae* isolates from diverse locations used in acquisition studies.

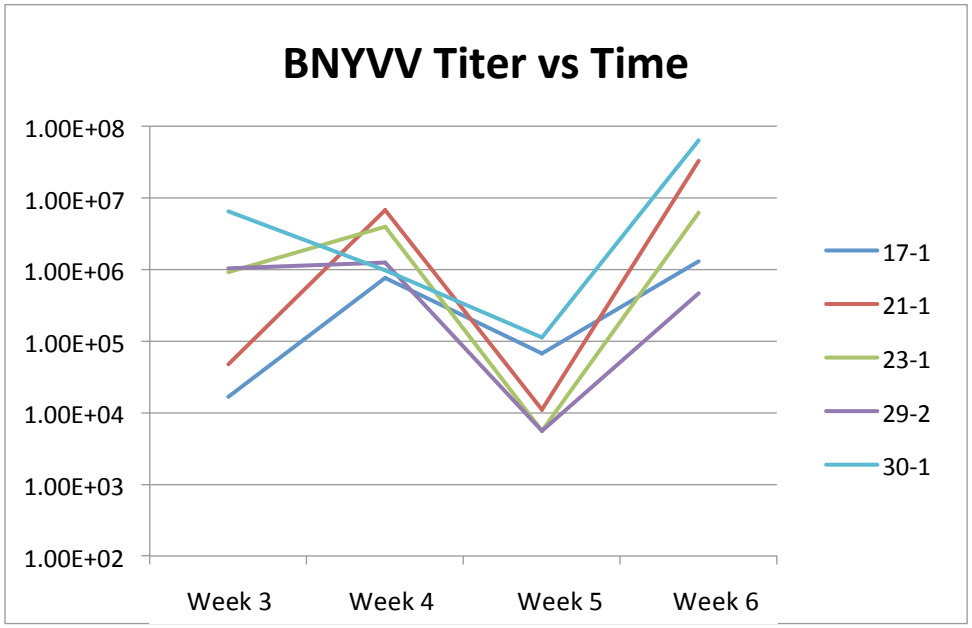

Figure 2. Changes in the level of BNYVV in extracted roots over a four week period among five treatments, each containing a different pure culture *P. betae* isolate. E=98.1%, R^2=1.0

Additional Information Relevant to Proposed Studies:

Field isolates of BNYVV were collected from locations known to carry traditional and *Rz1* resistance-breaking variants of BNYVV in both southern Minnesota and Imperial Valley, California during the summer of 2014. Samples were specifically selected from these regions because the two regions are believed to have some of the highest prevalence of resistance-breaking forms of BNYVV in the US, and the regions have very different types of growing conditions. BNYVV was baited from soil using standard BNYVV soil testing methods by growing seedling sugarbeets in the soil (Figure 2). At the conclusion of these tests, roots were collected, tested by ELISA for the presence of BNYVV, and if positive by ELISA (antibody-based test used to confirm virus infection), were used to inoculate *Chenopodium quinoa* plants. BNYVV produces lesions on *C. quinoa*, and if these lesions are isolated from one another, each isolate represents an individual infection source. This is a means of obtaining several distinct pure culture isolates from each growing region. Once lesions develop they were collected and used for transmission to wild beet (*Beta macrocarpa*), which can easily be infected systemically by BNYVV. Once each isolate is increased by propagation in *B. macrocarpa*, tissue can be stored for future use. These isolates will eventually be introduced into our standard pure culture *Polymyxa betae* isolate (Tracy, CA isolate) using methods described in Objective 1, and evaluated in standard rhizomania soil tests to compare virus accumulation in a series of Rz1 and Rz2 sugarbeet resistance sources described in the 2015 proposal. This will establish baseline infection aggressiveness scores for each isolate. So far about half of the new isolates have been processed to single local lesion isolates. The remaining samples are in various stages of baiting and transmission to C. quinoa. The initial isolates should be characterized genetically (sequencing of relevant regions of the virus genome) by May 2015, with all isolates characterized by the end of 2015.

Figure 4. Seedling sugarbeets being used to bait BNYVV from soil (Note: Similar strategy used for transmission and acquisition studies in Objectives 1 & 2).

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