EVALUATIONS OF DIFFERENTIAL ACQUISITION AND TRANSMISSION OF BNYVV BY *POLYMYXA BETAE* ISOLATES FROM US PRODUCTION REGIONS

Project Leader: William M. Wintermantel, Research Plant Pathologist, USDA-ARS, Salinas, CA

Collaborator: Frank Martin, Research Plant Pathologist, USDA-ARS, Salinas, CA

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 plasmodiophorid organism 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 that can survive in the field for many decades (**Fig. 1**). 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).

Studies are examining pure culture *P. betae* isolates from sugarbeet production regions, including several from the Western Sugar production region for differences in their ability to transmit BNYVV to sugarbeet. In preliminary 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tissue by real time PCR were modified to screen *Beta* germplasm for possible resistance to *P. betae* (H-Y. Liu, unpublished) as a follow-up to earlier studies by Barr et al. (1995). Although methods for this initial study were later found to need some improvement, results of those initial real-time PCR quantification assays for *P. betae* showed great variation for titer of the fungal vector among wild beet germplasm sources, including *Beta patelaris*, *Beta procumbens*, and *Beta webbiana*, the latter of which had low levels of *P. betae* in initial studies; this is in agreement with previous findings (Paul et al. 1992, 1993). Commercial hybrids varied greatly for titers of *P. betae* in preliminary tests, with ‘Angelina,’ a variety highly resistant to BNYVV, being most susceptible to accumulation of *P. betae*. This result was supported by microscopic examinations in which ‘Angelina’ had the most cystosori embedded in the roots of all the sugarbeet varieties examined. Based on those initial studies we developed new methods to more accurately determine levels of both *P. betae* and BNYVV from sugarbeet roots in a manner that allows determination of virus load relative to amount of *P. betae*. This facilitates our current project to find out if differences exist among *P. betae* isolates for their ability to carry and transmit BNYVV to sugarbeet, and is also allowing us to examine the rate at which BNYVV increases in a *P. betae* population. This is important in ongoing parallel studies on identification of resistance to *P. betae* in sugarbeet germplasm, as well as for management of BNYVV and its vector in the field.

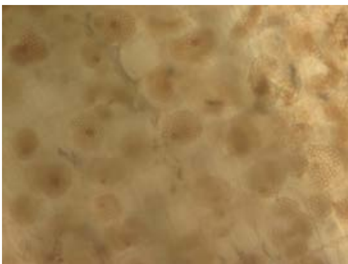

Figure 1. Light microscopy of *P. betae* cystosori visualized in sugarbeet roots. Cystosori are the clusters of spherical particles shown in root cells below. In this photo, there are cystosori in most of the cells on the surface of the root.

Objectives for 2015:

1. Complete studies on differences in acquisition of BNYVV by *P. betae* isolates collected from several US sugarbeet production regions.

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

3. Examine relationship between aggressiveness of pure culture BNYVV isolates on resistance sources for use in germplasm differentiation and evaluation.

Summary of Project to Date:

During the project immediately preceeding this one, methods for quantification of BNYVV levels using real time (quantitative) RT-PCR were developed that 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. Methods for quantification of *P. betae* levels using real-time (quantitative) RT-PCR were developed by the Frank Martin Lab (collaborator). Originally, methods for *P. betae* quantification were based on previously established methods (such as studies by Kingsnorth et al. 2003; Ward et al., 2004); however, it became clear that those methods were not reliable for the diverse *P. betae* isolates from wide ranging locations being evaluated in these studies. Therefore new methods were established by Dr. Martin's lab during the course of this project (Hogan, Smith, and Martin, unpublished), which were validated in spring 2015.

Polymyxa betae isolates were collected during the first year and a half of this project from most of the major sugarbeet production regions in the United States, including California, Colorado, Idaho, Minnesota, Nebraska, Oregon, and Wyoming. 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. Single cystosori (clusters of resting spores) were isolated and increased from each of these sources by propagation in sugarbeet to obtain what we call "single cystosorus" or pure culture isolates. These single cystosorus isolates were evaluated using the RT-PCR system described above for detection of soil-borne viruses, as well as antisera against *Beet oak leaf virus*, for which sequence data is not available to determine if any viruses were present. Any pure culture isolates containing viruses were removed from future experiments because we were interested in working with virus-free isolates only that would allow us to introduce our own virus isolate. In addition, field soil samples for collection of novel *Rz1* resistance-breaking isolates of BNYVV were obtained from fields symptomatic for rhizomania planted with *Rz1* sugarbeet during the summer of 2014 from the Southern Minnesota and the Imperial Valley, California production regions; the two regions with arguably the greatest prevalence of *Rz1*-resistance breaking BNYVV isolates. Sugarbeets were used to bait BNYVV from the soil and single lesion isolates were prepared from several samples. Results provided a range of variants for use in downstream studies.

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

In the fall of 2014 we began full-scale experimentation, and have continued this throughout the 2015 project year. Some adjustments to methods were made over the course of experiments. Once each pure culture *P. betae* isolate had been sufficiently increased and *P. betae* levels determined, wild beet (*Beta macrocarpa*) was planted into foam cups containing each soil with a different pure isolate of *P. betae*. *B. macrocarpa* plants were inoculated at the two to four 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 this plant more efficiently than in sugarbeet. This allowed BNYVV to move readily into root tissues where *P. betae* is active. Some pots of each *P. betae* isolate containing *B. macrocarpa* plants remained uninoculated as controls for each *P. betae* isolate. Co-cultivation of virus and vector was maintained for a period of 6 weeks in multiple foam cups per isolate.

At six weeks, seedling roots were carefully washed from plants in one cup and the entire root system was homogenized and ground to a fine powder with liquid nitrogen, then immediately transferred to a supercold freezer at -80C for storage prior to nucleic acid extraction and evaluation for presence of BNYVV in roots. Remaining pots were pooled and used in subsequent transmission experiments (described below). Initial plans under this objective were to monitor accumulation of BNYVV in extracted *P. betae* cystosori (resting spores) over time, but our methods to isolate cystosori from sugarbeet (*Beta vulgaris*) or wild beet (*B. macrocarpa*) roots were unsuccessful, and were found to be contaminated with plant tissue. Therefore results could not be considered reliable; therefore we did not pursue this approach further. Because we were unable to separate cystosori from root tissue, acquisition studies were modified to focus on confirmation that BNYVV had been acquired by *B. macrocarpa* roots and that cystosori were present on those roots. Total RNA was extracted from 100ug frozen *P. betae* root tissue for each isolate using established methods. The RNA 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 to confirm levels of BNYVV. Subsequent studies described later in this report illustrate revised approaches used for monitoring of BNYVV in *P. betae* populations.

Transmission efficiency of BNYVV varies among *P. betae* isolates.

We determined that BNYVV increase within populations of *P. betae* varies by fungal population, including from sources collected from areas near one another. Transmission experiments were conducted over 6 week periods per experiment in Conviron PGC15 growth chambers using standardized conditions of 6 h days, 24°C, and approximately 220 uM m^{-1} s^{-2} light. Experiments were conducted in standardized soil (Monterey Bay Academy sandy loam soil mixed 50:50 with sterile builders' sand). Each isolate to be tested was introduced separately to this soil mix. Each isolate used (except negative controls) had been previously been tested to confirm presence of BNYVV following introduction of the virus to the isolate through inoculation of *B. macrocarpa* plants as described above. Approximately 50 seeds of a standard BNYVV susceptible (rz1) sugarbeet variety were planted into *P. betae* infested soil carrying BNYVV in new 280 ml styrofoam cups held in new plastic saucers. Multiple cups were used for each soil in each experiment so that one cup could be harvested each week, beginning at 3 weeks post-planting (sampled at 3, 4, 5, and 6 weeks). Seeds were covered with sand and watered with gentle misting daily to facilitate germination. Following germination, 50 ml sterile water was added to the saucers daily to prevent wilting using individual watering pitchers dedicated for each treatment. This allows water to absorb into soil and the soil to dry over the course of the day, promoting root infection by *P. betae*. This may seem like excessively frequent watering, but have determined over the course of these studies that if seedlings get too dry they become stressed or die and this affects the experimental results.

At 3, 4, 5, and 6 weeks, roots were washed free of soil, immediately ground in liquid nitrogen, and stored at -80 until all collections are completed. At the end of the 6 week collection, all samples were processed at the same time for extraction of nucleic acid (DNA and RNA). DNA and RNA were prepared separately from 100 ug of each ground sample (split same sample, one part for DNA extraction the other for RNA) for use in determining *P. betae* and BNYVV levels, respectively. Quantitative RT-PCR was performed on RNA for measuring BNYVV titers and quantitative PCR was performed on DNA for measuring *P. betae* levels. Results allow comparison of amount of *P. betae* present relative to the amount of BNYVV present.

After BNYVV is first introduced to soil using inoculation of *B. macrocarpa*, and soil is subsequently planted with sugarbeet, the titer of BNYVV as measured in sugarbeet roots often accumulates slowly over time, with BNYVV either undetectable or detected at a low levels following the first planting of sugarbeet into these soils. This is not true for all *P. betae* isolates, because some appear to accumulate virus much more quickly resulting in relatively high levels of BNYVV after even a single planting of sugarbeet (**Fig. 2**). Levels of *P. betae* were generally fairly consistent among isolates, with an initial spike in *P. betae* populations at the 3 week sampling point (first sampling), with lower levels in weeks 4, 5, and 6 (**Fig. 3**). It is unclear why this pattern occurs. These results demonstrate clear variability for virus transmission efficiency among *P. betae* isolates. Figure 2 only shows isolates for which measurable virus accumulation was detected. Several isolates had levels below the monitoring threshold and will be included in subsequent tests once titers increase with sequential plantings of sugarbeet.

In **Fig. 2**, most isolates were transmitted BNYVV to sugarbeet at low rates following initial introduction of BNYVV to *P. betae*. This included the Tracy, CA isolate, which is very aggressive at forming cystosori in sugarbeet. In contrast, three isolates including two from Scottsbluff, NE, and one from Moorhead, MN, transmitted BNYVV very

well, resulting in high accumulation of BNYVV even as early as 3 weeks post-planting. To the best of our knowledge, all factors were maintained uniformly in these experiments. Titers of *P. betae* followed the same trend for all isolates tested (**Fig. 3**), including both the aggressive Moorhead 17-2 isolate and several isolates with lower transmission rates. This indicates some *P. betae* isolates are likely much more efficient than others for transmission, or alternatively, a higher proportion of the *P. betae* population may be infected for these isolates. Interestingly, both isolates from the Scottsbluff, NE area produced similar results with high transmission rates across all sampling time points. In contrast, the two isolates from Moorhead, MN produced vastly different results, with Sample 17-2 having an exceptionally high transmission rate comparable to the Scottsbluff isolates, whereas the other Moorhead sample (17-1) had an exceptionally low rate of virus transmission at or near the minimum threshold of detection. Like Moorhead 17-1, several samples (not shown) had very low or undetectable rates of transmission in initial experiments following introduction of BNYVV to *P. betae*. Most *P. betae* isolates (Renville, "Mitch", Nampa, and Tracy) transmitted at a low rate as would be expected for the first planting into soils containing *P. betae* to which BNYVV had been recently introduced.

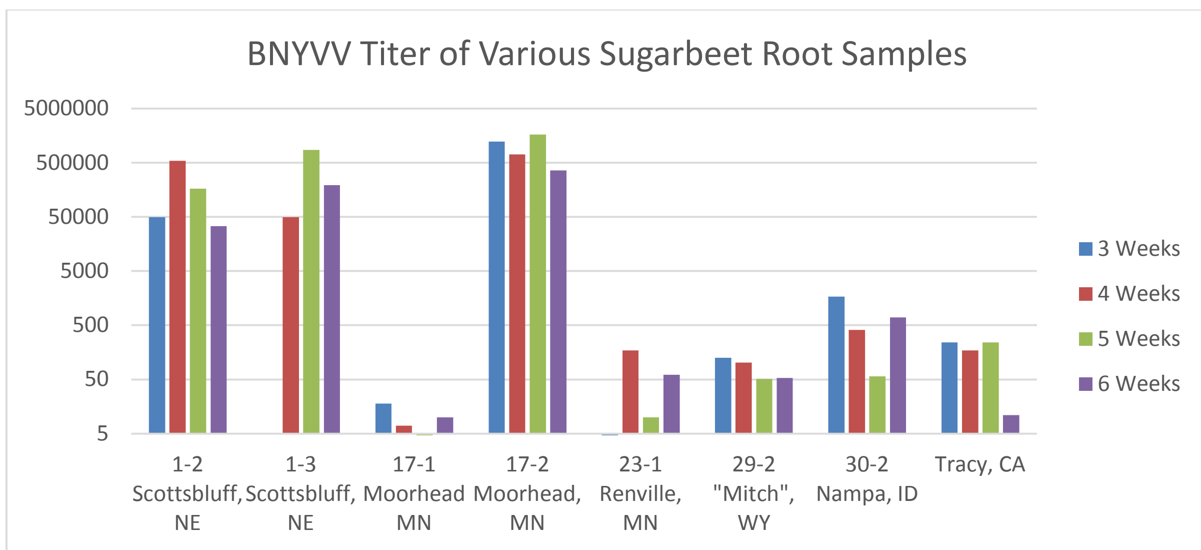

Figure 2. Titer of BNYVV in sugarbeet roots following transmission by separate pure culture (single cystosorus) isolates of *Polymyxa betae* at 3-6 weeks post-planting. Isolate numbers are listed above location where isolate was collected.

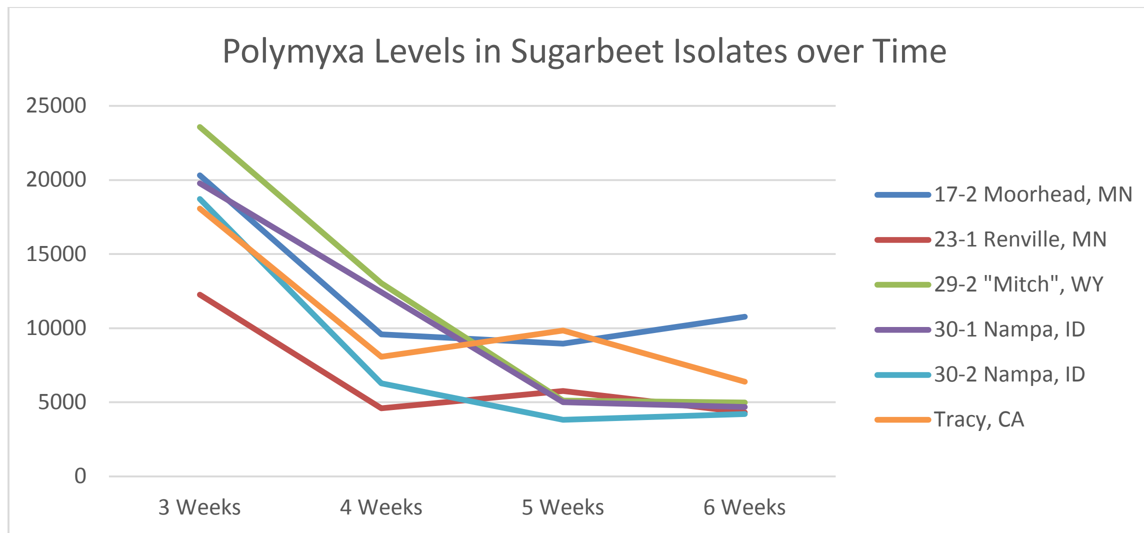

Figure 3. Titer of pure culture (single cystosorus) isolates *Polymyxa betae* in sugarbeet roots from BNYVV transmission experiments sampled at 3-6 weeks post-planting.

In fields, BNYVV increase within a population of *P. betae* generally occurs somewhat slowly over a few crops with infection spreading gradually through a field following cultivation and movement by water. This is similar to what we observe with what we refer to as "low transmission rate isolates" in our experiments. However, our results also suggest that some *P. betae* isolates accumulate and efficiently transmit BNYVV relatively quickly after introduction of the virus to soil carrying virus-free *P. betae*, indicating that some "high transmission rate isolates" may be either more efficient at accumulating BNYVV and distributing it throughout the fungal population, or that these isolates are just better at transmitting BNYVV than other isolates. The latter would be more similar to what one would observe for insect-transmitted viruses. This may occur with some *P. betae* populations in fields as well, but methods were not previously available to document this. Further studies will be needed to clarify the cause of differences in virus transmission rates among *P. betae* isolates. It is possible these differences may be related to genetic differences among the *P. betae* isolates themselves (the isolates are being characterized genetically by Dr. Martin's lab) that may make them more efficient at accumulating BNYVV or transmitting the virus to sugarbeet, but we also cannot yet rule out other factors and are still working to develop a strategy to specifically measure virus levels in individual cystosori, although this is a challenging task. This work is continuing in 2016 through completion of studies comparing isolates using the same single BNYVV source, but will also begin with alternate sources of genetically distinct BNYVV isolates later this year to determine if variation exists for transmission of different BNYVV isolates by individual *P. betae* sources (the reciprocal of current experiments).

Related Studies:
Near isogenic lines of sugarbeet differing for individual Rz genes were developed by Dr. Kelley Richardson (ARS Salinas) over the past few years through a separate project. These lines contain not only five known Rz genes, but also some additional sources of resistance from the Salinas program that have not been well characterized. Some of our single cystosorus (pure culture) isolates are being used to evaluate combined resistance to both BNYVV and *P. betae* using this material. Results are pending, but results are not sufficiently complete for presentation. These studies will continue as will further studies on whether transmission patterns differ depending on the genetics of the BNYVV isolates used in experiments.

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