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

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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 plasmodiophorid protozoon 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tissue by real time PCR were modified to screen *Beta* germplasm for possible resistance to *P. betae* (H-Y. Liu, unpublished).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.

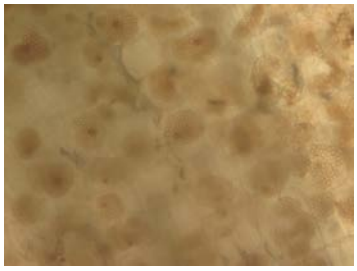


Fig. 1. Light microscopy of *P. betae* cystosori visualized in sugarbeet roots. Cystosori are the clusters of spherical particles shown in root cells below. In this photo, there are cystosori in most of the cells on the surface of the root.

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 have been developed 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 the previous project. New methods for quantification of *P. betae* levels have been evaluated by the Martin Lab (Co-PI) and appear to have sufficient specificity, although validation of the new methods for *P. betae* quantification is continuing, and will need to be evaluated with more *P. betae* isolates. In addition, a new hydroponic method is being developed for production of large amounts of specific single spore isolates of *P. betae* for use in experimentation. Furthermore, these pure culture isolates of *P. betae* collected from sugarbeet production regions around the U.S. have been isolated and increased over the past year. Studies are in progress to evaluate differential acquisition and transmission of virus by these isolates.

Specific Accomplishments for 2013:

Isolation and increase of single "spore" isolates of Polymyxa betae from U.S. sugarbeet regions

Most of the year was spent purifying and increasing pure culture isolates of *P. betae* collected from soil samples throughout the United States. Single cystosori (long-term resting spores of *P. betae*) were isolated from several isolates over the past year and a half. These isolates were then increased and evaluated to determine if any viruses were present using standard methods for virus detection (RT-PCR) (Table 1). Isolates contaminated with BNYVV, BSBMV, or BSBV were eliminated.

Table 1. Evaluation of presence or absence of soil-borne sugarbeet viruses in single cystosorus isolates of *Polymyxa betae* collected from western U.S. sugarbeet fields*.

Location	Sample	BNYVV		BSBV	BSBMV
Nampa, ID	**30-1**	N		N	N
	30-2	N		N	N
Weld, Co	**9-1**	N		N	N
Greeley, Co	**21-1**	N		N	N
Declo, ID	**19-1**	POS		N	N
Nyssa, OR (NE Oregon)	**35-1**	POS		N	N
Moorhead, Mn	**17-1**	N		N	N
	17-2	N		N	N
Scottsbluff, NE	**1-1**	N		N	N
	1-2	N		N	N
	1-3	N		N	N
Renville, Mn	**23-1**	N		N	N
	23-2	N		N	N
Mitch, WY	**29-1**	N		POS	N
	29-2	N		N	N
	29-3	N		N	N

- All samples evaluated by RT-PCR using primers specific for each virus. N = Negative for all viruses, POS = positive detection of the virus indicated. More than one sample number indicates multiple isolates from a location. *This is only a subset of all pure culture P. betae isolates evaluated and being propagated*.

Development of hydroponic methods for experiments with P. betae and sugarbeet

Once single cystosorus (pure culture) isolates were confirmed virus free, each was propagated further to increase quantities to levels suitable for studies. This takes considerable time because we are essentially taking a single cystosorus (one of the clusters of "balls shown in a single cell in Figure 1) and allowing it to reproduce to a level where we can inoculate multiple pots of beets at the same time. This takes multiple cycles and several months, and is done by raising sugarbeets in foam cups with a soil-sand mixture as is used in standard rhizomania soil-tests. In order to increase pure culture isolates for specific experiments, the Martin Lab (collaborator) is developing a hydroponic system for *P. betae* propagation.

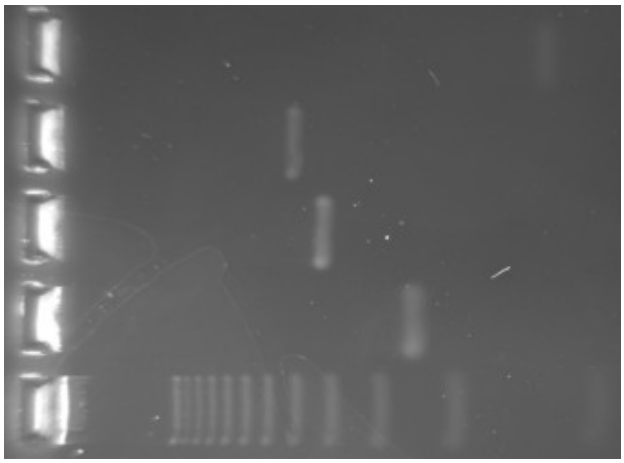
Seedlings are first germinated in a soil free system before being planted into the hydroponic system. The seeds need to be treated with a bleach-detergent solution before being placed on wet paper towels (Figure 2). Otherwise they rot and die shortly after being placed in the hydroponic system.

Figure 2. Comparison of bleach treatments to eliminate damping off of beet seedlings in hydroponic culture used for propagation of *P. betae*. The image shows the results of a trial comparing 0.5% **(left)** and 2% **(right)** bleach solutions with seeds being treated for various lengths of time (5 minutes on bottom, 10 minutes in the middle, and 15 minutes on top). The seeds treated with 2% solution did not germinate very well, nor did the seeds treated with 0.5% solution treated for 15 minutes; however the 0.5% solution for 5 and 10 minutes seemed to germinate quite well.

Quantitative detection of P. betae and soil-borne viruses

Methods for standard and quantitative detection of all three major soil-borne viruses of sugarbeet (BNYVV, BSBMV, and BSBV) were developed during the previous project (See 2012 project report). Similarly, previously published studies had developed methods for quantitative detection of *P. betae*, the

vector of these viruses (Kingsnorth et al., 2003) using a method targeting the RNA of *P. betae*. That study measured *P. betae* RNA, whereas our studies are targeting the genomic DNA of *P. betae*. While it may not seem like this would matter, it does. The Kinsgnorth primers are suitable for RNA detection, but when DNA is the target we found these primers can amplify sugarbeet sequences in addition to those of *P. betae*. Therefore it was necessary to develop or identify new primers. A different study (Ward et al., 2004) published shortly after the Kingsnorth study used a type of qPCR probe known as a TaqMan probe, and evaluation to date suggests the Ward primers may be effective for our studies although confirmation is still in progress. To date we have been able to amplify expected bands from purified *P. betae* DNA (Fig. 3), and have confirmed these by sequencing. Optimization of parameters for qPCR is continuing, but results are promising.



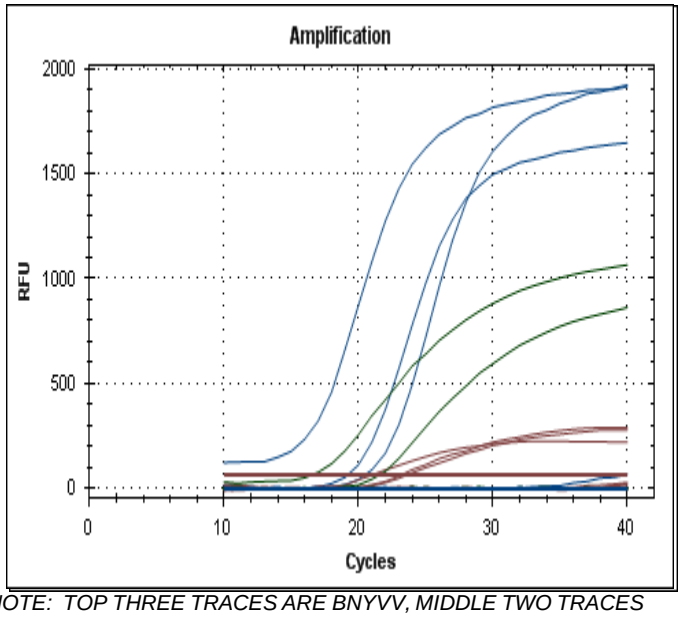
Lane Key:
1. Water negative control
2. Sugarbeet internal control (135 bp)
3. Polymyxa betae primers (108 bp)
4. P. betae primers (63 bp)
5. 25 bp DNA Ladder to determine band sizes

Figure 3. The above gel demonstrates that our PCR primers amplify specific regions of the *P. betae* genome (chromosomal DNA) and that our quantitative PCR (qPCR) probe targets are working properly. DNA from bands was purified and sequenced to confirm bands were the expected *P. betae* target sequences.

Methods for quantitative detection of soil-borne viruses has also been applied to *P. betae* single cystosorus isolates. This was described and shown in the 2014 proposal as it will be an important method during the upcoming project year. I am including the same figure here because it shows differential amplification of viruses from single RT-qPCR reactions. The curves rising above the baseline in the chart in Figure 4 indicate presence of virus and demonstrate clean differential detection and quantification of each virus from purified *P. betae* isolates. This method was developed during the previous project year, but is now being used to evaluate isolates.

Figure 4. Differential detection of soil-borne viruses from single cystosorus isolates of P. betae.

Target	Sample ID	Cq
BNYVV	1-3	0.00
BNYVV	17-2	0.00
BNYVV	19-1	22.05
BNYVV	29-1	0.00
BNYVV	BNYVV -1	20.56
BNYVV	BNYVV CDNA	14.88
BNYVV	BSBMV -1	0.00
BNYVV	BSBMV CDNA	0.00
BNYVV	BSBV -1	0.00
BNYVV	BSBV CDNA	0.00
BNYVV	NTC	0.00
BNYVV	Reagent Blank	0.00
BSBMV	1-3	0.00
BSBMV	17-2	0.00
BSBMV	19-1	0.00
BSBMV	29-1	0.00
BSBMV	BNYVV -1	0.00
BSBMV	BNYVV CDNA	0.00
BSBMV	BSBMV -1	22.73
BSBMV	BSBMV CDNA	18.13
BSBMV	BSBV -1	0.00
BSBMV	BSBV CDNA	0.00
BSBMV	NTC	0.00
BSBMV	Reagent Blank	0.00
BSBV	1-3	0.00
BSBV	17-2	0.00
BSBV	19-1	0.00
BSBV	29-1	23.33
BSBV	BNYVV -1	0.00
BSBV	BNYVV CDNA	0.00
BSBV	BSBMV -1	0.00
BSBV	BSBMV CDNA	0.00
BSBV	BSBV -1	23.77
BSBV	BSBV CDNA	21.21
BSBV	NTC	0.00
BSBV	Reagent Blank	0.00



NOTE: TOP THREE TRACES ARE BNYVV, MIDDLE TWO TRACES ARE BSBMV, AND BOTTOM THREE TRACES ARE BSBV. THE SAMPLES THAT WERE POSITIVE ARE HIGHLIGHTED (See Table 1 above). Controls for each virus are shown by highlighted boxes within the left-hand column.

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

Single cystosorus isolates of Polymyxa betae have been isolated and have been/are being increased during the past year 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 is 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 will be inoculated at the two leaf-stage with a single local lesion (pure culture) isolate of BNYVV pathotype A, the traditional form of BNYVV. *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 will allow 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. The first acquisition experiments began in December 2013, since development of pure culture isolates took considerably longer than expected.

The first experiment, comparing differences in acquisition of BNYVV by single spore isolates of P. betae from throughout the U.S., will be completed in February 2014, and the first sampling will occur on January 13 with additional samplings in subsequent weeks. Roots will be washed free of soil, and *P. betae* cystosori purified using methods previously established by the Martin Lab. **DNA and RNA will be prepared separately from each root and/or cystosori sample (same sample will be used for both) for use in determining *P. betae* and BNYVV levels, respectively.** Quantitative RT-PCR will be performed on RNA for measuring BNYVV titers, and quantitative PCR will be performed on DNA for measuring *P. betae* levels. Results will allow comparison of amount of *P. betae* present to the amount of BNYVV present in the *P. betae*. Acquisition experiments will be 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 will allow comparison across experiments based on standards used as controls.

Once the first acquisition study is complete, *P. betae* isolates carrying BNYVV will be used for evaluation of virus transmission efficiency to sugarbeet, again in sequentially replicated experiments conducted in growth chambers.

Literature Cited:

Abe, H and Tamada, T. 1986. Association of beet necrotic yellow vein virus with isolates of *Polymyxa betae* Keskin. Ann. Phytopathol. Soc. Japan. 52:235-247.

Atallah, Z.K. and Stevenson, W.R. 2006. A methodology to detect and quantify five pathogens causing potato tuber decay using real-time quantitative polymerase chain reaction. Phytopathology 96:1037-1045.

Barr, KJ, Asher, MJC, and Lewis, BG. 1995. Resistance to *Polymyxa betae* in wild *Beta* species. Plant pathol. 44:301-307.

Bilodeau, G.J., Koike, S.T., Uribe, P. and Martin, F.N. 2012. Development of an assay for rapid detection and quantification of Verticillium dahliae in soil. Phytopathology 102: 331-343.

Kaufmann, A, Koenig, R, and Lesemann, DE. 1992. Tissue printing-immunoblotting reveals an uneven distribution of beet necrotic yellow vein and beet soil-borne viruses in sugarbeets. Arch. Virol. 126:329-335.

Keskin, B. 1964. *Polymyxa betae* n. sp., a parasite in the roots of *Beta vulgaris* torunefort, particularly during the early growth of the sugar beet. Arch. Mikrobiol. 19:348-374.

Kingsnorth, CS, Kingsnorth, AJ, Lyons, PA, Chwarszczynska, DM and Asher, MJC. 2003. Real-time analysis of *Polymyxa betae* GST expression in infected sugar beet. Mol. Plant Pathol. 4:171-176.

Larson, R.C., Hill, A.L., Wintermantel, W.M., Fortis, L.L., and Nunez, A. 2008. Proteome changes in sugarbeet in response to Beet necrotic yellow vein virus. Physiological and Molecular Plant Pathology. DOI 10.1016/j.pmpp.2008.04.003

Liu, H-Y and Lewellen, RT. 2007. Distribution and molecular characterization of resistance-breaking isolates of *Beet necrotic yellow vein virus* in the United States. Plant Dis. 91:847-851.

Liu, H-Y, and Lewellen, RT. 2008. Suppression of resistance-breaking *Beet necrotic yellow vein virus* isolates by *Beet oak-leaf virus* in sugar beet. Plant Disease 92:1043-1047.

Papayiannis, L.C., Hunter, S.C., Iacovides, T., and Brown, J.K. 2010. Detection of *Cucurbit yellow stunting disorder virus* in Cucurbit Leaves Using Sap Extracts and Real-time TaqMan® Reverse Transcription (RT) Polymerase Chain Reaction (PCR). J. Phytopathology 158: 487-495.

Paul, H, Henken, B, De Bock, ThSM, Lange, W. 1992. Resistance to *Polymyxa betae* in *Beta* species of the section P*rocumbentes*, in hybrids with *B. vulgaris* and in monosomic chromosome additions of *B. procumbens* in *B. vulgaris*. Plant Breed. 109:265-273.

Paul, H, Henken, B, Scholten, OE, De Bock, ThSM, Lange, W. 1993. Variation in the level of infection with *Polymyxa betae* and its effect on infection with *Beet necrotic yellow vein virus* in beet accessions of the sections *Beta* and *Corollinae*. In: Proceedings of the 2nd Symposium of the International Working Group on Plant Viruses with Fungal Vectors, Montreal, Canada, pp.133-136.

Tamada, T. and Baba, T. 1973. *Beet necrotic yellow vein virus* from rhizomania-affected sugarbeet in Japan. Ann Phytopathol Soc Japan 39:325-332.

Ward, L.I, Fenn, M.G.E., and Henry, C.M. 2004. A rapid method for direct detection of Polymyxa DNA in soil. Plant Pathology 53: 485-490.

Wisler, GC, Lewellen, RT, Sears, JL, Wasson, JW, Liu, H-Y, and Wintermantel, WM. 2003. Interactions between *Beet necrotic yellow vein virus* and *Beet soilborne mosaic virus* in sugar beet. Plant Dis. 87:1170-1175.

Wisler, GC, Liu, H-Y, Duffus, JE. 1994. *Beet necrotic yellow vein virus* and its relationship to eight sugarbeet furo-like viruses from the U.S.A. Plant Dis. 78:995-1001.