2003 Sugarbeet Research and Extension Reports. Volume 34, Page 309-311

GENETIC VARIABILITY AMONG ISOLATES OF BNYVV AND BSBMV AND VIRULENCE TO CURRENT RHIZOMANIA RESISTANT CULTIVARS

C.M. Rush, K. Maxson, and K. Steddom
Texas Agricultural Experiment Station, P.O. Drawer 10, Bushland Texas 79012

Beet Necrotic Yellow Vein Virus (BNYVV) and Beet Soil Borne Mosaic Virus (BSBMV) are closely related viruses found throughout the growing regions of Minnesota and North Dakota. BNYVV infection typically results in rhizomania, which causes reductions in extractable sucrose and yield. Both viruses are vectored by the fungus, *Polymyxa betae* and occupy similar ecological niches. Because of this, the possibility of viral recombination has become an issue. A new virus that can infect BNYVV resistant cultivars like BSBMV, but also cause severe damage like BNYVV would be a threatening combination. In 2002, a strain of BNYVV that could overcome resistance was found in California's Imperial Valley. This new strain puts all of the BNYVV-resistant sugar beet crops at risk because nearly all of them rely on the same gene (Holly) for resistance. By understanding the variability that exists in natural and emerging populations of these viruses, we can evaluate the risk to Minnesota and North Dakota sugar beet growers.

New sources of resistance to BNYVV are needed. Relying on one gene for resistance puts intense selective pressure on BNYVV to overcome it. Traditional methods for determining BNYVV resistance can be inaccurate and time consuming. We hypothesize that real-time PCR could be useful in identifying genetic resistance to BNYVV in cultivars and breeding lines. It could quantify resistance not solely based on the *Rz* gene in a shorter amount of time with a higher degree of accuracy.

Results

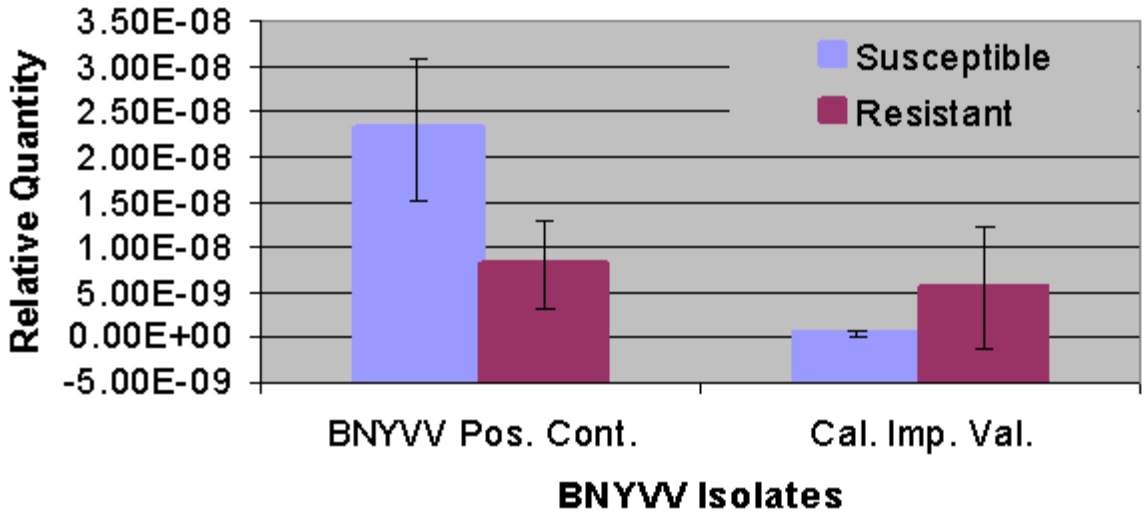
Objective 1. Quantify genotypic variation among isolates of BNYVV and BSBMV. Rhizosphere soil from sugar beets with root or foliar symptoms of BNYVV was collected from sugar beets fields in California (CA), Idaho (ID), Michigan (MI), Minnesota (MN), North Dakota (ND) and Texas (TX). BNYVV strain-type controls were obtained from dried sugar beet root tissue infected with BNYVV from Italy (A-type), Japan (A-type), Germany (1 and 2, both B-types), and France (P-type). Rhizosphere soil from sugar beets with foliar symptoms of BSBMV was collected from Colorado (KM, RC, EA), Minnesota (BS, LN, S), North Dakota (Fargo, SH) and Texas (SS, PL, HK). All soil samples were bioassayed in the green house and viral RNA was isolated from baited sugar beet roots.

PCR Primers were designed to amplify regions of BNYVV and BSBMV RNA1, RNA2, RNA3, RNA4, and RNA5 (BNYVV only) with PCR. Variability was detected in the replicase portion of the 237 KDa protein (RNA 1), in isolates MN, TX, ID, CA, Italy (A-type control), and Germany1 (B-type control). The replicase protein is responsible for viral replication, so it is possible but unlikely that the variations observed have a significant effect on viral replication because we were able to purify all isolates from sugar beet plants with relative ease. Double bands and size variation were observed in products amplified from the end of the 25 KDa protein (RNA 3). The 25 KDa protein is associated with leaf symptoms and root proliferation and it's possible that the genomic variations observed in these isolates affect their symptomology.

None of the isolates studied contain an RNA 5 capable of detection with the primers used, indicating that the isolate from the Imperial Valley (CIV) is either lacking an RNA 5 or it carries one that is significantly different from those observed in the past. Little or no variation was observed in BNYVV RNA 2 and RNA 4 in the isolates studied. When the BSBMV readthrough protein (RNA 2) was amplified, deletions of approximately 400bp were observed in isolates RC, SH, and PL. Sequence data from the readthrough rt-PCR products confirms 459 bp, 407 bp, and 363 bp deletions in these isolates respectively. The BNYVV 75 KDa readthrough protein has been associated with virus transmission and it is possible that transmission is affected by the deletions in these isolates.

Objective 2. Measure disease tolerance among BNYVV tolerant cultivars by quantitative PCR. Thirty sugar beets, fifteen MonoHy 9155, susceptible to BNYVV, and fifteen Crystal R207, were planted in sand and grown for 10 days. Five seedlings from each variety were then vortexed in 5 ml of a buffer solution with a standard BNYVV isolate, or buffer with the aggressive "CIV" isolate. Seedlings were repotted and grown for another 10 days. Viral RNA was extracted from seedling roots and hypocotyls, and reverse-transcribed to cDNA. The cDNA was amplified with real-time quantitative PCR using primers pairs specific for BNYVV coat protein. As hypothesized, standard BNYVV infection was significantly higher in the susceptible variety (MonoHy 9155) than in the resistant variety (Crystal 207) (Fig. 1). However, infections with the CIV isolate were low in both lines but not significantly different between the susceptible and resistant cultivars (Fig. 1). This response is what one would expect from a virus isolate that had overcome resistance of a resistant cultivar. Seedlings vortexed with PBS by itself were not infected (data not shown). The preliminary results of this study supported our hypothesis that real time PCR could be useful in identifying genetic resistance to BNYVV in cultivars and breeding lines. It could quantify resistance not solely based on the *Rz* gene.

Figure 1. Compared to the susceptible cultivar, MH 9155, virus concentration of the standard BNYVV isolate was significantly reduced in the rhizomania resistant cultivar. However, the CIV isolate was not reduced in the resistant cultivar and virus titer was no different than in the susceptible line. This technique would be an excellent method of quantifying resistance to the new CIV isolate in newly developed germplasm.



Objective 3. Relate virus genotype (BNYVV and BSBMV) to incidence and severity of infection in resistant and susceptible sugar beet cultivars. Soil from the Imperial Valley of California was planted with seed of the rhizomania resistant cultivar Beta 4776 in order to bait out the "new BNYVV strain", which was designated as the California Imperial Valley "CIV" isolate. ELISA was used to confirm BNYVV infection in these plants and total RNA was isolated. An RNA gel was run to tentatively determine whether the CIV isolate contained a RNA 5 species but no RNA 5 was apparent. Total RNA was reverse-transcribed to cDNA for PCR analysis and subsequent sequencing. Nucleotide sequences of BNYVV from GenBank were compared with the nucleotide sequence of our CIV isolate. PCR Primers specific for the coat protein of BSBMV were used to confirm that RNA isolations from plants infected with the "CIV" isolate of BNYVV did not contain BSBMV RNA. The PCR products generated covered the majority of sequence of each BNYVV RNA. CIV sequences from each individual RNA species were assembled and analyzed using the Lasergene software and the BLAST algorithm (http://www.ncbi.nlm.nih.gov/) (Table 1, 2).

Table 1. Nucleotide sequence comparison of PCR products from isolate "CIV" with their closest matches from Genbank

			Closest matching sequence in GenBank			Sequence from "CIV" isolate compared with matching sequence in GenBank	
RNA species	# of PCR products	Sequence length	Accession #	"Type" classification	Length	Nucleotide position	%nucleotide similarity
RNA 2	6	4417 bp	D84411	Type A	4609 bp	137-4553 nt	98%
RNA 3	2	1346 bp	AF197558	Type A	1725 bp	117-1463 nt	99%
RNA 4	2	1129 bp	AF197552	Type A	1416 bp	116-1244 nt	99%

The nucleotide sequence of the CIV isolate was ≥ 98% identical to previously published BNYVV sequences. This means that development of a specific probe for this new virus "strain" is likely to be very difficult if not impossible. Furthermore, most of the single nucleotide polymorphisms (SNPs), i.e. differences, observed were insignificant. However some resulted in amino acid substitutions (Table 2). One such substitution ("C" to "T") at nucleotide position 1011 (relative to accession # AF197552) of RNA 4 replaced a tyrosine residue with a histidine residue. Histidine residues are often found in the active sites of enzymes and this change could possibly be responsible for virulence of the CIV isolate to cultivars previously resistant to rhizomania. However, additional research must be conducted to verify this observation.

Table 2. Amino acid sequence comparison of putative proteins from isolate "CIV" with their closest matches from GenBank.

BNYVV Protein	RNA species	Nucleotide position*	% amino acid similarity*
Coat Protein (CP)	RNA 2	145-708 nt	100%
Read-throughProtein (RT)	RNA 2	145-2217 nt	99.4%
42K Protein	RNA 2	2130-3284 nt	99.7%
13K Protein	RNA 2	3284-3640 nt	99.2%
15K Protein	RNA 2	3624-4022 nt	98.5%
14K Protein	RNA 2	4034-4423 nt	98.4%
25K Protien	RNA 3	421-1080 nt	99.5%

31K Protein	RNA 4	348-1196 nt	99.6%

Objective 4 (not included in project proposal). Detection and quantification of Beet Necrotic Yellow Vein Virus in soil with real-time quantitative PCR. Traditionally, detection of BNYVV in soil requires a bioassay in which *P. betae* is baited with sugar beet roots, and BNYVV is then detected by ELISA or conventional RT-PCR methods. Inoculum density can be estimated using the Most-Probable-Number technique which is time-consuming and inaccurate. *P. betae* has been detected in soil using real-time quantitative PCR, which suggests this method could also be used to detect and quantify BNYVV in soil. Total RNA was isolated from soil infested with viruliferous *P. betae* and was reverse transcribed to cDNA. Primers and probes designed for the coat protein gene of BNYVV were used to detect and quantify BNYVV using real-time PCR. Results indicate this method may be useful in determining inoculum density in field soils used for sugar beet production.

Objective 5 (from second proposal) Validation of a remote sensing model for detection of Rhizomania. During 2000 and 2001 we conducted a study to determine if Rhizomania could be detected with remote sensing methods. Through this work we found that Rhizomania reduced chlorophyll and carotenoid and increased betacyanin content of beet leaves. We also confirmed previous studies that showed reduced foliar nitrogen in beets infested with BNYVV. A manuscript detailing this work was prepared and published in the journal Phytopathology. Since this was the first such attempt at this type of study, our experimental design was limited to best determine the spectral differences between healthy and diseased sugar beets. Due to this, there was low variability in nitrogen content in the healthy beets in this study. When considering this along with the finding that Rhizomania reduces foliar nitrogen, we felt it best to validate our findings under a large range of nitrogen levels. This work was initiated in 2003 with the cooperation of John Lamb and Mark Bredehoeft in the Southern Minn region and with Larry Smith and Joseph Giles in the American Crystal region. Work in the American Crystal Region focused on a single experimental filed in Glyndon, Minnesota. This field suffered from a wet spring and consequently had a great deal of Aphanomyces root rot. Fusarium tip rot was also found in this field. We felt these diseases might confound our results and chose to focus our research elsewhere. In the Southern Minnesota region we sampled three fields that were part of a nitrogen management study. All of the fields had patchy rhizomania, with average levels of infestation between 32 and 38 percent in the susceptible variety ACH 999. The resistant variety Beta 4811 had average infestations ranging from 10 percent to 50 percent, while Vanderhave 46177 ranged from 10 to 46 percent. Foliar and canopy spectral readings were taken in late August and late September just prior to harvesting tare samples. Nitrate and amino nitrogen is being analyzed on the beet tissue, and total nitrogen and nitrate nitrogen on the leaf tissue. This will be a very large data set, requiring some time for statistical analysis.

Previous work. A manuscript detailing the usefulness of a radiometer for quantification of Cercospora leaf spot has been prepared and submitted for review to the journal Plant Disease. We found that the radiometer had much higher precision than visual estimates, but that visual estimates were more sensitive at differentiating treatments. For studies where results need to be compared across multiple sites, the radiometer will provide the best data. However, at this point in time we feel radiometric assessments should always be accompanied by visual estimates.