Prevalence and Distribution of Beet Necrotic Yellow Vein Virus Strains in North Dakota and Minnesota

John J. Weiland,¹ Kathrin Bornemann,^{1,2} Jonathan D. Neubauer,¹ Mohamed F. R. Khan,^{2,3} and Melvin D. Bolton^{1,2,†}

¹ United States Department of Agriculture – Agricultural Research Service, Edward T. Schafer Agricultural Research Center, Fargo, ND

² Department of Plant Pathology, North Dakota State University, Fargo, ND

³ Department of Plant Pathology, University of Minnesota, St. Paul, MN

Abstract

Beet necrotic yellow vein virus (BNYVV) is the causal agent of rhizomania, a disease of global importance to the sugar beet industry. The most widely implemented resistance gene to rhizomania to date is *Rz1*, but resistance has been circumvented by resistance-breaking (RB) isolates worldwide. In an effort to gain greater understanding of the distribution of BNYVV and the nature of RB isolates in Minnesota and eastern North Dakota, sugar beet plants were grown in 594 soil samples obtained from production fields and subsequently were analyzed for the presence of BNYVV as well as coding variability in the viral P25 gene, the gene previously implicated in the RB pathotype. Baiting of virus from the soil with sugar beet varieties possessing no known resistance to rhizomania resulted in a disease incidence level of 10.6% in the region examined. Parallel baiting analysis of sugar beet genotypes possessing *Rz1*, the more recently introgressed *Rz2*, and with the combination of *Rz1* + *Rz2* resulted in a disease incidence level of 4.2, 1.0, and 0.8%,

respectively. Virus sequences recovered from sugar beet bait plants possessing resistance genes *Rz1* and/or *Rz2* exhibited reduced genetic diversity in the P25 gene relative to those recovered from the susceptible genotype while confirming the hypervariable nature of the coding for amino acids (AAs) at position 67 and 68 in the P25 protein. In contrast to previous reports, we did not find an association between any one specific AA signature at these positions and the ability to circumvent *Rz1*-mediated resistance. The data document ongoing virulence development in BNYVV populations to previously resistant varieties and provide a baseline for the analysis of genetic change in the virus population that may accompany the implementation of new resistance genes to manage rhizomania.

Keywords: viruses, cultivar resistance, BNYVV, *Rz1*, *Rz2*, resistance breaking

Rhizomania, caused by the beet necrotic yellow vein virus (BNYVV; family *Benyviridae*, genus *Benyvirus*), is a leading disease of sugar beet worldwide (Rush et al. 2006). First reported in Italy in the early 1950s (Canova 1959), rhizomania spread from central and southern Europe to eastern and northern Europe and the Middle East within a few decades (Asher 1993; McGrann et al. 2009). Rhizomania was found in Japan in 1965 (Tamada and Baba 1973), in China in 1978 (Gao et al. 1983), and in the United States in 1983 (Al Musa and Mink 1981; Duffus et al. 1984). Disease symptoms were first described in detail by Tamada and Baba (1973) and include an extreme lateral root proliferation, wineglass-shaped beets of reduced size, and brown discoloration of the vascular tissue. Yellowing of leaf veins can be found occasionally in infected plants, whereas a more common uniform yellowing of the leaves often is observed from a distance as discrete patches composed of several infected plants

within a field. Such yellow patches are typically surrounded by ostensibly uninfected or nonsymptomatic green plants, which is the basis for the colloquial expression of “blinkers” to describe these infected plants in the field. Rhizomania is transmitted by the plasmodiophoromycete *Polymyxa betae* (Fujisawa and Sugimoto 1977), and the movement of infested soil by contaminated machinery and climatic factors is the main route of disease transfer between fields and regions. Viable resting spores of *P. betae* can persist for more than 15 years in desiccated soil samples (Abe and Tamada 1986) and in fields lacking sugar beet production for nearly two decades (Payne and Asher 1990). No chemical treatments to manage *P. betae* are registered; consequently, rhizomania currently is managed through the use of resistant cultivars.

Although sugar beet hybrids with some measure of tolerance to rhizomania had been known in Europe for decades (Biancardi et al. 2002), the first characterized source of resistance to BNYVV was discovered by the Holly Sugar Company in the mid-1980s (Lewellen 1997), the basis for which was shown to be conditioned by a single dominant gene designated *Rz1* (Scholten et al. 1996). A second resistance gene, *Rz2*, subsequently was discovered in *Beta maritima* (Scholten et al. 1999) and has been increasingly utilized in sugar beet growing areas with high rhizomania pressure. Within the last 15 years, *Rz1*-breaking strains have been observed in several sugar beet growing areas throughout the world, including the Red River Valley in the northern plains of the United States (Liu et al. 2005; Rush and Acosta-Leal 2007). Infrequent reports of isolates compromising *Rz2* resistance exist in the literature, most if not all restricted to studies in which virus was baited from infested soil using resistant sugar beet varieties as opposed to the recovery of virus from symptomatic, field-grown sugar beet (Acosta-Leal et al. 2010a; Bornemann et al. 2015; Kutluk Yilmaz et al. 2018; Liu and Lewellen 2007).

BNYVV is a positive-sense, single-stranded RNA virus with a genome divided into four or five RNAs, each encapsidated in a separate rod-shaped particle (Richards and Tamada 1992; Tamada 2016). Although RNAs 1 to 4 are required for field infections, nonvectored

Current address for K. Bornemann: Nordzucker AG, Zuckerfabrik 3, 31249 Hohenhameln, Germany

†Corresponding author: M. D. Bolton; Melvin.Bolton@ars.usda.gov

J. J. Weiland and K. Bornemann contributed equally to this work.

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infections can be produced using RNAs 1 to 3 on sugar beet and some laboratory hosts. Relevant to studies on strain and pathotype discrimination in BNYVV is the broad grouping of isolates as being A-, B-, or P-type based upon sequence variation between the types, including that of the viral coat protein gene, and the presence (for P-type) of a fifth genomic RNA encoding the supplemental virulence protein P26. The A-type of the virus characterizes U.S. isolates to date with the caveat that the virus and the distribution of the recognized types continues to change worldwide (Chiba et al. 2011; Schirmer et al. 2005). Following the discovery of isolates inducing varying degrees of disease on susceptible sugar beet and virus strains capable of overcoming *Rz1* resistance, viral RNA reassortment studies clearly demonstrated that the major factors determining isolate aggressiveness mapped to RNA 3 (in strains containing four components [Koenig et al. 1991; Tamada et al. 1999]) and additionally RNA 5 (in strains containing five components, currently only found outside of the United States [Tamada et al. 1999]). The RNA 3-encoded P25 protein is considered to be both a pathogenicity factor and a major determinant of aggressiveness in BNYVV. In several cases, the ability for BNYVV to circumvent *Rz1*-mediated resistance has been shown to map to the P25 gene (Chiba et al. 2011; Gilmer 2016; Tamada 2016). Evidence further has accumulated that a highly variable “tetrad” of amino acids (AAs) in P25 (AAs 67 to 70) appeared to be involved in conferring the RB pathotype to BNYVV. For example, a single nucleotide substitution encoding an A67 to V67 AA change in the RNA 3-encoded P25 protein was shown to be sufficient to produce a strain of BNYVV capable of defeating the *Rz1* resistance gene (Acosta-Leal et al. 2010b; Koenig et al. 2009).

In eastern North Dakota and western and central Minnesota, approximately 260,000 ha of sugar beet is planted every year, which corresponds to over 50% of sugar beet planted in the United States (Bangsund et al. 2012). First identified in this growing region in 1996 (Wisler et al. 1997), rhizomania has since spread to most reaches of the producing area and further has been observed to generate strains of BNYVV that break *Rz1* resistance. Nevertheless, the distribution and status of RB isolates of BNYVV in this region remain poorly characterized, necessitating systematic and sustained research on a molecular level regarding the virus subtypes and their relevance to disease induction and severity. The objectives of this study were to identify locations in the sugar beet growing regions of North Dakota and Minnesota that harbor BNYVV (as indicated by the ability of a susceptible sugar beet variety to contract rhizomania disease from sampled soil) and further to identify strains able to induce detectable infection in varieties with resistance mediated by *Rz1*, *Rz2*, or *Rz1* + *Rz2*. Additionally, our objectives were to carry out sequence analysis of the P25 gene for all BNYVV-positive samples to produce a baseline for virus variation in this growing region as it relates to the breaking of currently deployed resistance to rhizomania disease.

Materials and Methods

Soil sampling. A total of 594 field samples were obtained throughout the sugar beet production areas of the Red River Valley and southern Minnesota by agriculturalists of American Crystal Sugar Co. (Moorhead, MN), Minn-Dak Farmers Cooperative (Wahpeton, ND), and Southern Minnesota Beet Sugar Cooperative (Renville, MN) in spring of 2014. Each field sample was a composite of four to six soils taken randomly throughout the designated field. As a follow-up in 2015, a total of 34 soil samples were obtained from the immediate perimeter around yellow-crowned “blinker” sugar beet plants ostensibly having rhizomania disease and deviating in this manner from green, healthy-looking plants within a field planted with a *Rz1*-possessing commercial variety. In both sampling years, soil samples were thoroughly mixed with sterile sand in a 1:10 ratio and were transferred into 250-ml plastic pots. Slow-release fertilizer (Multicote; Sungro Horticulture, Agaway, MA) was added following the manufacturer’s instructions.

Plant growth. The sugar beet varieties used in this study were SLC4-K583-G1C (susceptible), B13A00001 (*Rz1*-mediated resistance), ES0002 (*Rz2*-mediated resistance), and B12A00155 (*Rz1* + *Rz2*-mediated resistance), which were kindly provided by

SESVanderHave (Tienen, Belgium). Sugar beet seeds were germinated in sterile sand and transplanted between the ages of 7 and 14 days old into 250-ml pots prepared as described above. For each soil sample, eight pots with two replications per treatment (sugar beet variety as treatment variable) were planted (8 to 10 seedlings per pot) and placed into trays. As a negative control, the same number of plants per treatment was planted into sterile sand. Seedlings of a susceptible genotype were planted into rhizomania-infested soil from the Imperial Valley of California, which served as a positive control. Plants were grown in the greenhouse under standardized conditions at 24°C (day)/18°C (night) with 8 h of supplemental light per day. To control damping-off diseases, plants were sprayed with the fungicide hymexazol (Tachigaren 70 WP; Sumitomo, Tokyo, Japan) at 90 g per 100,000 plants. Water was added directly as needed into the trays. Five weeks after seedling transfer to the infested soil, plants were harvested, and a root sample consisting of five to seven seedlings was taken from each pot. Roots were washed under running tap water, dried, and stored at –80°C until further processing.

Molecular analysis. Root samples from susceptible plants were ground for enzyme-linked immunosorbent assay (ELISA) with a homogenizer (Sartorius, Göttingen, Germany) using phosphate buffered saline/Tween/polyvinylpyrrolidone buffer in a 1:15 (root mass/buffer volume) ratio. Qualitative double antibody sandwich ELISA with BNYVV-specific antibodies was conducted as described in Pferdmenges et al. (2009). Root samples were considered infected with BNYVV when ELISA values exceeded the mean plus three times standard deviations of the negative controls. Positive samples were taken for RNA extraction using TRIzol reagent (Life Technologies, Grand Island, NY) following the manufacturer’s protocol. A one-step reverse transcription polymerase chain reaction (RT-PCR) kit (Promega, Madison, WI) was used to amplify the RNA3-encoded P25 gene sequence. The PCR reaction mix contained 12.5 µl of 2× PCR Master Mix, 10 µl of nuclease-free water, 100 pmol of forward primer (MDB-1175: 5′-CCTACGGCCGCATTG TAAAA-3′), 100 pmol of reverse primer (MDB-1176: 5′-TTT GGTGTAATCGTCCGAAGA-3′), and 0.5 µl of RNA (final volume 25 µl). Primers were designed based on conserved regions of BNYVV P25 sequences retrieved from GenBank. Negative control (no RNA) and BNYVV-positive control reactions were included in each PCR. The thermal cycler program for PCR was as follows: reverse transcription at 45°C for 45 min, denaturation at 95°C for 2 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 1 min, and extension at 68°C for 2 min, and a final extension was performed at 68°C for 7 min. Amplicons were purified with SureClean (Bioline, Taunton, MA) according to the manufacturer’s protocol and sequenced (MCLAB, San Francisco, CA) using primers MDB-1175 and MDB-1176. Sequencing trace files representing the entire P25 gene were evaluated using the Contig Express tool within the Vector NTI software package (Invitrogen, Carlsbad, CA) for the possibility of mixtures of BNYVV strains in any one sample. Amplicon sequence traces of P25-coding sequence in which a competing nucleotide peak value was 25% or greater of the primary nucleotide trace value was designated “variable.” Variable amplicons were cloned into the pGEM-T Easy vector (Promega) for independent clone sequence analysis. For each of the 34 samples containing such mixed haplotypes, 10 pGEM-T Easy colonies containing inserts were sequenced in both directions using M13 forward and reverse primers. Consensus sequences were generated and analyzed using Vector NTI. Representative P25 gene sequences have been deposited in GenBank under accession numbers MK241660 to MK241675.

Root samples obtained from *Rz1*, *Rz2*, and *Rz1* + *Rz2* plants were likewise processed if the sample was ELISA positive in susceptible plants. *Rz1*, *Rz2*, and *Rz1* + *Rz2* plant samples were subsequently processed following the same procedure mentioned above.

Mapping of positive samples. The origin of all samples from susceptible, *Rz1*, *Rz2*, and *Rz1* + *Rz2* plants detected as positive for BNYVV by means of ELISA and RT-PCR was drawn on maps using Google MyMaps with color coding to designate sugar processing cooperative regions (red, American Crystal Sugar; blue, Minn-Dak

Farmers Cooperative; green, Southern Minnesota Beet Sugar Cooperative).

Results

Identification of soils harboring BNYVV with a bait plant test using four sugar beet genotypes. Soil samples were harvested from 594 sites in eastern North Dakota and Minnesota in 2014 (Supplementary Fig. S1) and used as a source of natural inoculum for the recovery of BNYVV on sugar beet genotypes with varying sources and combinations of genetic resistance. For the susceptible treatments, 63 of 594 (10.6%) sites showed infection. As anticipated, the number of sites harboring isolates capable of circumventing *Rz1* resistance was higher (25 sites; 4.2%) when compared with isolates capable of breaking *Rz2* resistance (six sites; 1.0%) or the combination of *Rz1* and *Rz2* (five sites; 0.8%). The sites containing BNYVV strains able

to cause disease on any of the four sugar beet genotypes were found throughout the growing regions of Minnesota and North Dakota (Fig. 1).

P25 sequence analysis in susceptible plants. ELISA-positive bait-plant samples were used for amplification of the BNYVV P25 gene followed by sequence analysis. Analysis of sequencing trace files revealed that 34 of the 63 samples contained multiple P25 gene sequence variants, indicating that these plant infections were composed of multiple BNYVV strains. Although there were up to three different AAs found in positions 42, 122, 140, and 142, variability between samples was the highest for AA position 67 and 68, the first two AAs making up the P25 hypervariable tetrad (Supplementary Table S1). Only one sample showed variability on position 69, whereas position 70 exhibited no variability in the susceptible genotype in this study.

Fig. 1. Collection sites of rhizomania-positive samples. Collection sites containing beet necrotic yellow vein virus strains able to cause disease on a rhizomania-susceptible variety (A) and varieties with resistance mediated by *Rz1* (B), *Rz2* (C), or *Rz1* + *Rz2* (D). Location color denotes sugar beet factory district: American Crystal Sugar Co. (red), Minn-Dak Farmers Cooperative (blue), and Southern Minnesota Beet Sugar Cooperative (green).

P25 sequence analysis in resistant plants. No BNYVV-positive samples from bait plants possessing *Rz1*, *Rz2*, or *Rz1* + *Rz2* contained P25 sequence ambiguity within the sample amplicon; therefore, no PCR product cloning was required. Nine P25 AA positions were variable among the *Rz1*-positive samples (Table 1). Sequence variability between samples was highest at position 67, which harbored an alanine in most cases (65%) and valine in the remainder of the samples. Although there was less variability at position 68, three AAs were identified at this position (Table 1). AA position 69 showed no variability, and 95% of the samples harbored a glycine at position 70. Furthermore, only positions 25, 31, and 210 also showed variability in comparison with the susceptible samples. AA polymorphism at positions 5, 171, and 212 was unique to *Rz1*-positive samples. Overall, the variability in the *Rz1* resistant plants was less than that observed in susceptible samples. The dominant tetrads detected in *Rz1* bait plant assays were ACHG (45%) followed by VCHG (35%), AHHG (10%), and ACHR and ALHG (both at 5%).

For *Rz2* bait plants that were ELISA positive, the variability at position 67 was lower than that observed in susceptible and *Rz1* resistant plants, with 83.3% of the samples showing an alanine at position 67 and only 16.7% a valine (Table 1). The dominant tetrads detected in these bait plants were ACHG (66.7%) followed by AHHG and VCHG (both at 16.7%). Finally, *Rz1* + *Rz2* resistant plants testing positive by ELISA in this study showed the least variability between the few infected plants detected. Variability was observed only at AA positions 21, 36, and 68 (Table 1), with the dominant tetrads recovered being ACHG (40%), AHHG (40%), and ALHG (20%).

P25 tetrad status in which resistance of multiple genotypes was compromised. Two locations (sample nos. 43 and 45) were identified as possessing BNYVV variants capable of inducing positive ELISA values in all varieties tested in this study (Table 2). In both cases, the tetrad identified in bait plants containing any resistance gene was also found in the susceptible bait plant (Table 2). Additionally, three other locations were positive in inducing infections in the susceptible genotype and at least two resistant bait plant genotypes. Unlike samples 43 and 45, the tetrad ACHR identified in *Rz1* bait plant tests in sample 157 was not detected in the susceptible plants (Table 2). In general, no tetrad was clearly associated with the ability to break resistance in any given resistance genotype used as plant bait.

BNYVV P25 haplotypes in soil collected from field “hot spots” in 2015. Prior to this study, the examination of RB isolates in the United States suggested that VCHG was the dominant RB tetrad found in diseased sugar beet plants harboring *Rz1* in the field. Because the data collected within the present study using bait plants in infested soil were only partly consistent with this conclusion, additional soil samples were collected in the 2015 growing season that specifically targeted soil (i) in fields concurrently planted to *Rz1*-possessing varieties and (ii) immediately adjacent to “blinker” infected beet plants.

Table 3 shows the P25 tetrad composition in susceptible sugar beet plants cultivated in specifically collected soil. Tetrads ACHG, VCHG, AFHG, and AHHG were found in 28.9, 23.7, 17.8, and 11.9% of all samples, respectively. Reported for the first time in any study of P25 variation, a TCHG tetrad composition was also found in these samples (17.8%). In *Rz1* resistant plants, ACHG was found in 58.3% of all samples, whereas VCHG was found in 41.7% of all samples (Table 3). In the *Rz2*-resistant plants, VCHG was the predominant tetrad (60%), whereas it was found in 50% of *Rz1* + *Rz2* bait plants (Table 3). ACHG was found in 40% of all *Rz2*-positive samples and 50% of all *Rz1* + *Rz2*-positive samples. Thus, only VCHG and ACHG were

Table 3. Beet necrotic yellow vein virus P25 tetrad compositions in sugar beet baited from targeted soil adjacent to “blinkers” in 2015

Resistance trait	Tetrad	% ^a
Susceptible	ACHG	28.9
	VCHG	23.7
	TCHG	17.8
	AFHG	17.8
	AHHG	11.9
<i>Rz1</i> bait	ACHG	58.3
	VCHG	41.7
<i>Rz2</i> bait	VCHG	60
	ACHG	40
<i>Rz1</i> + <i>Rz2</i> bait	VCHG	50
	ACHG	50

^a Percentages based on the number of sample locations harboring a specific P25 tetrad sequence.

Table 1. Polymorphic sites in P25 from beet necrotic yellow vein virus strains identified in the sugar beet varieties with single and combined resistance genes^a

Resistance trait	AA	AA position										
		5 ^b	21	25	31	36	67	68	70	171	210	212
<i>Rz1</i> bait	Primary AA	L (95.0)	–	D (95.0)	T (95.0)	–	A (65.0)	C (85.0)	G (95.0)	M (93.8)	Q (93.8)	P (93.8)
	Substitute 1	F (5.0)	–	N (5)	A (5.0)	–	V (35.0)	H (10.0)	R (5.0)	I (6.3)	L (6.3)	S (6.3)
<i>Rz2</i> bait	Substitute 2	–	–	–	–	–	–	L (5.0)	–	–	–	–
	Primary AA	–	T (83.3)	D (83.3)	–	–	A (83.3)	C (83.3)	–	M (66.7)	–	–
	Substitute 1	–	M (16.7)	N (16.7)	–	–	V (16.7)	H (16.7)	–	I (16.7)	–	–
<i>Rz1</i> + <i>Rz2</i> bait	Substitute 2	–	–	–	–	–	–	–	–	V (16.7)	–	–
	Primary AA	–	T (60.0)	–	–	C (80.0)	–	C (40.0)	–	–	–	–
	Substitute 1	–	M (40.0)	–	–	R (20.0)	–	H (40.0)	–	–	–	–
	Substitute 2	–	–	–	–	–	–	L (20.0)	–	–	–	–

^a Percentages based on 25, six, and five ELISA-positive samples in sugar beet varieties possessing resistance genes *Rz1*, *Rz2*, and *Rz1* + *Rz2*, respectively. Excludes amino acid (AA) positions that had two or fewer strains exhibiting an AA polymorphism.

^b Numbers inside parenthesis indicate % of strains possessing that particular AA.

Table 2. Predominant tetrads in locations associated with ability to circumvent major beet necrotic yellow vein virus resistance genes

Resistance trait	Sample no.				
	43	45	157	330	350
Susceptible	AHHG/ACHG	ACHG/AHHG	ACHG	ACHG/AHHG	VCHG
<i>Rz1</i> bait	ACHG	AHHG	ACHR	ACHG	VCHG
<i>Rz2</i> bait	ACHG	AHHG	ACHG	No infection	VCHG
<i>Rz1</i> + <i>Rz2</i> bait	AHHG	AHHG	No infection	ACHG	No infection

found in the resistant plants, whereas five different tetrads were found in susceptible plants.

Discussion

The current investigation was undertaken to determine incidence and distribution of rhizomania in the sugar beet production regions of Minnesota and eastern North Dakota and to ascertain the extent to which RB isolates exist within this region. Unlike sugar beet diseases induced by fungi for which fungicide use is available to complement genetic resistance in the crop, genetic resistance remains the only cost-effective and registered means for the control of rhizomania, highlighting the concern over RB isolates of BNYVV. Using soil samples collected randomly from all sugar beet production districts within Minnesota and eastern North Dakota, virus strains potentially capable of circumventing resistance were captured in the greenhouse by bait plants harboring no resistance to BNYVV in parallel with those harboring commercially implemented rhizomania resistance genes *Rz1* and *Rz2*. Because the BNYVV P25 protein is a known pathogenicity factor and has been shown in the United States and elsewhere to be associated with the ability to break *Rz1*-mediated resistance, sequence variability within the gene encoding this protein was analyzed, including the AAs 67 to 70 composing the “tetrad” region of the protein previously associated with the RB effect and considered to be “hypervariable” relative to sequences exhibiting high variation in other plant and animal viruses (Klein et al. 2007; Schirmer et al. 2005). The results of the analysis would have practical implications for rhizomania resistance breeding efforts. For example, sugar beet breeders may be inclined to continue reliance on *Rz1*-mediated resistance to provide protection in this growing region if P25 variability is low (Janzac et al. 2009). By contrast, extensive variability in P25 may suggest that alternative sources of resistance should be identified and prioritized as a proactive means to augment *Rz1* and *Rz2* in ensuring management of rhizomania.

The results confirm the wide distribution of rhizomania throughout the eastern North Dakota and western and central Minnesota sugar beet growing regions (Fig. 1). An incident rate in susceptible bait capture of ~11% suggests a regional inoculum density lower than that detected in other rhizomania-infested locations around the world, even as positive samples were obtained from all factory districts covering the entire production region. For example, Mouhanna et al. (2002) sampling 115 locations in Syria and Yilmaz et al. (2016) monitoring production fields in Turkey found that the disease is readily detected in production areas with 48 and 38%, respectively, of all samples testing positive. The higher incidence possibly reflects the closer proximity of these countries to seed exchange networks within Europe, because seed harboring microbial contaminants is considered a main route by which new pathogens emerge in a region (Elmer 2001; Heijbroek 1988), and the southern regions of the continent have been affected by rhizomania disease since the 1950s (Canova 1959). By contrast, rhizomania can be considered a recent introduction to the northern plains of the United States relative to well-established diseases such as *Rhizoctonia* root and crown rot (Bolton et al. 2010) and *Cercospora* leaf spot (Weiland and Koch 2004) and as such may still be adapting to local soils and environment. It is noteworthy, moreover, that varieties possessing *Rz1*-based resistance were introduced within 3 years of the discovery of the disease as a crop protection measure (Larry Ronsberg, American Crystal Sugar, personal communication), which may in turn have slowed inoculum build-up. With the emergence of RB strains on predominantly *Rz1*-expressing hybrids (Liu and Lewellen 2007; Liu et al. 2005), the use of dual-source resistant genotypes became increasingly important. The first *Rz1* + *Rz2* resistant varieties were introduced in the region in 2010 to secure crop yields even as the combination of both *Rz1* and *Rz2* in first-generation commercial hybrids was associated with a yield penalty in the absence of disease. Considered collectively, these factors may have resulted in a lower regional rhizomania incidence than anticipated at the study’s inception, although specific subregions nevertheless may exhibit markedly higher incidences. That occasional disease “escapes”, in which the susceptible variety was not infected despite growing in BNYVV-

infested soil, may have contributed to a lower estimate of BNYVV incidence in our study cannot be ruled out.

The P25 tetrad type ACHG was found to predominate in samples obtained throughout the study in infections of sugar beet plants expressing *Rz1* (Tables 1 and 2) even as this tetrad has not widely been considered a confirmed RB type under production field conditions. This observation diverges somewhat from those noted by Acosta-Leal et al. (2008) and Liu and Lewellen (2007) in their analysis of U.S. isolates of BNYVV while corroborating the variation in P25 of A-type strains observed around the globe (Chiba et al. 2011). Prior to this work, the A67 to V67 AA change predominated among *Rz1*-associated RB isolates within the examined region (Acosta-Leal et al. 2010b). Additionally, the A67 to V67 mutation has been associated with A-type RB pathotypes of BNYVV in the western United States (Acosta-Leal et al. 2010b; Liu and Lewellen 2007) as well as Europe and the Middle East (Chiba et al. 2011; Schirmer et al. 2005). Subsequent investigations, primarily involving bait-plant capture of BNYVV isolates from sampled soil in controlled environments, have demonstrated the ability for isolates other than those encoding the V67 change to overcome *Rz1* (Bornemann et al. 2015; Chiba et al. 2011; Galein et al. 2018; Liu and Lewellen 2007). Interestingly, the presence of V67 in P25 is associated with reduced fitness of the virus even as it conferred RB and high aggressiveness to *Rz1*-encoding sugar beet (Bornemann and Varrelmann 2013), a general observation noted frequently in studies of pathogen virulence and the factors or effectors underlying these traits (Fraile et al. 2011; Moreno-Pérez et al. 2016). That soils in the southern Minnesota production region appear to harbor proportionally higher levels of V67-characterized tetrads in P25 compared with other soils in the region (not shown) may indicate that additional changes have occurred elsewhere in the viral genome in this subpopulation that act to partially stabilize the virus against this fitness penalty.

Given the prior evidence and subsequent focus on the V67 mutation in studies within the United States regarding RB strains of *Rz1* leading up to this work, it was remarkable that the tetrad type ACHG predominated when baited from infested soils using resistant genotypes in the current investigation. Although ACHG has emerged in baiting approaches over the past decade as a potential RB tetrad type, only recently has it been observed as a haplotype associated with RB in field-harvested, symptomatic sugar beet (Rekoske et al. 2015). Indeed, ACHG isolates were considered to represent the “wild-type” non-RB reference variant against which V67-encoding RB strains were compared (Acosta-Leal et al. 2010a), a result potentially highlighting the dynamic nature of virus and/or vector diversity and interactions at play in the adaptation of BNYVV to new resistance genes implemented in the crop. It has been noted in other virus-host systems that host or environmental changes reducing the ability for the virus to multiply effectively can lead to the emergence of multiple virus genotypes capable of restoring full or partial ability for the virus to multiply to its wild-type state (Kashiwagi et al. 2014). The observations and evidence combined from studies both within and outside of the United States, therefore, indicate that specific isolates detected within this study other than those encoding V67 in P25 are capable of inducing rhizomania disease in the field on resistant varieties, either alone or in combination with each other, and that other regions of the BNYVV genome may play a role as new RB types emerge.

Studies in Europe (Bornemann and Varrelmann 2013) and observations in the United States have indicated that sugar beet genotypes possessing *Rz1* + *Rz2* are able to limit the accumulation of isolates possessing the V67 AA in P25 although novel RB isolates nevertheless may be developing to this resistance gene combination (Galein et al. 2018; Kutluk Yilmaz et al. 2018; Liu and Lewellen 2007). Results from the present study indicate that isolates possessing the tetrad VCHG and ACHG accumulated in *Rz2* and *Rz1* + *Rz2* plants in the greenhouse at a sufficient level to produce a positive ELISA reaction and additionally to represent similar proportions of the infecting BNYVV population, respectively, a development that warrants future monitoring (Table 3). It is intriguing that exposure of the virus population to *Rz2* additionally selected for a T21M exchange in P25

(Table 1), a substitution present infrequently in other P25 sequences archived in the GenBank sequence database (not shown). It is possible that this change would condition the ability for the cellular translational apparatus to initiate the synthesis of the P25 protein at this methionine, thereby impacting P25 expression and structure in a manner influenced by the presence of *Rz2*. Extensive molecular analyses indicate that expression of P25 is highly nuanced and that, in addition to triggering the resistance response, the protein exhibits multiple activities including the modulation of host transcription and the direct interaction with cellular proteins (Gil et al. 2018; Gilmer 2016).

As a consequence of the widespread use of a single principal resistance gene (*Rz1*) and a second supporting resistance gene (*Rz2*) more recently deployed, high selection pressure is exerted on the BNYVV population. Rhizomania pressure and the incidence of RB variants appear to be more skewed toward the southern half of the tested region relative to the overall concentration of sugar beet acres in Minnesota and North Dakota, reflecting those subregions in which rhizomania was first reported along with the warmer average growing season promoting subsequently greater cycling of infection. It is probable that the abundant variation in the BNYVV P25 tetrad in addition to other potential changes in the genome reflects efforts to evade host resistance deployed in commercial sugar beet varieties to combat the disease. It is less likely, therefore, that a single molecular pathotype will arise under these conditions, because multiple variants may in fact be capable of evading the resistance/surveillance system of host plants in this situation (Harrison 2002; Moreno-Pérez et al. 2016). More definitive evidence for the role of the different variants of BNYVV in circumvention of resistance awaits the testing, with infectious cDNA clones of the virus, of genetically pure isolates in which only nucleotide substitutions anticipated to be involved in the RB pathotype are altered.

Even as rapid advances have been made to introduce naturally occurring resistance (Capistrano-Gossman et al. 2017) or otherwise develop artificial sources of genetic resistance to rhizomania, a great deal remains unknown regarding the many interactions at play in this soilborne disease and the dynamics of vector, virus, and the multiple isolates involved (Andika et al. 2016; Syller and Grupa 2016; Tromas et al. 2014). The evolution of greater virulence in BNYVV toward commercial sugar beet genotypes is of great interest to producers and likely is balanced against survival in (and potential multiplication within [Lubicz et al. 2007]) the resident population of *P. betae*, the genetic and physiological diversity of which remains poorly characterized. Moreover, recent reports have indicated potentially novel alternative hosts for *P. betae* and BNYVV, a finding that may foster additional diversity within the virus population as it adapts to different hosts that support sublethal or greater levels of multiplication of the virus (Hugo et al. 1996; Kutluk Yilmaz et al. 2016; Mouhanna et al. 2008). In this respect, it is anticipated that future investigations regarding the population structure of BNYVV and its plasmodiophorid vector might employ whole genome sequencing in an effort to draw potential associations between tetrad sequence status in P25 and additional modifying changes throughout the virus and vector genomes in the generation of RB strains. These factors highlight the ongoing research needed to monitor and control rhizomania of sugar beet, a complex disease of historical and continuing threat to world beet sugar production.

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