ETIOLOGY OF RHIZOMANIA IN FIELDS PLANTED TO RESISTANT CULTIVARS

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The incidence of rhizomania in fields planted to resistant cultivars in Minnesota and North Dakota has steadily increased during the last 2 - 3 years. In 2006, we continued to investigate factors that were involved, or not involved, with development of rhizomania. Numerous ideas as to the cause of rhizomania in resistant cultivars have been put forth and the most commonly suggested include 1) problems with seed quality during production of hybrid seed, 2) soil physical or chemical factors, 3) inoculum density of the pathogen, and 4) emergence of new resistance-breaking strains of BNYVV. Research on all four of these was conduced and some were eliminated as factors involved with break down of resistance.

METHODS

Seed Purity. Studies were conducted to determine whether rhizomania in fields planted to resistant cultivars could be associated with seed purity issues. Our two basic questions were whether the plants exhibiting severe rhizomania symptoms possessed the *Rz1* resistance gene, and secondly, whether the observed distribution of rhizomania in the field was consistent with what one would expect if a portion of the seed did not have the *Rz1* resistance gene.

Blinker Rz1 Study. Plants for this study were collected from individual grower's fields located near Crookston, Moorhead, and Renville, MN. Plants exhibiting the typical fluorescent yellow foliage that is associated with rhizomania, and asymptomatic plants, were collected from each field. Multiple locations in each field were sampled, and 10 to 20 beets were collected from each location. The sampled plants were individually rated for rhizomania severity on 0 – 4 scale with 0 = no symptoms and 4 = extremely severe root stunting, constriction, and bearding. Foliage from each plant was collected and scanned using a hyperspectral radiometer to quantify the degree of leaf chlorosis, and root and rhizosphere soil was collected so *Beet necrotic yellow vein virus* (BNYVV) could be baited from individual plants if deemed necessary. After roots were rated and rhizosphere soil collected, symptomatic and asymptomatic plants were separated and those in each group were bulked for sucrose determination. Subsamples of root tissue from each plant were tested for BNYVV by the enzyme-linked immunosorbent assay (ELISA) test and leaf tissue was sent to the various cooperating seed companies to test for the presence of the *Rz* gene. Collected data was subjected to a variety of statistical tests to determine whether the *Rz1* gene was actually present in severely diseased plants, and, if it was present, whether it had a significant effect on disease severity and percent sucrose. Over 500 individual beets were included in this study.

Spatial Analysis Study. Four fields were selected to test whether the incidence and distribution of rhizomania in fields planted to resistant varieties was random, and possibly a result of planting a percentage of seed that did not possess the *Rz* gene. Within each field, four areas were sampled. Each sampling area was fifty feet x 20 rows. The number of symptomatic plants and the total number of plants in each sampling area was determined. Approximately 15

symptomatic plants and 15 asymptomatic sugar beets were collected to determine root yield and sugar differences between healthy and diseased plants, within the sampled area. The sugar beets were rated for rhizomania severity on a scale from 0-4, and the diseased and healthy plants were bulked separately at each location. This gave a total of four paired samples for each field. Each sample was processed for sucrose content and yield. BNYVV was assayed by ELISA on feeder root tissue of the taproot.

A white tarp measuring approximately 3ft by 10ft was placed at each sample location so that it could be identified in aerial photography (Fig. 1A). Immediately after the fields were sampled on the ground, digital images were acquired at an altitude of approximately 1700 ft mean sea level (800 ft above ground) using fixed wing aircraft. The images were acquired with an Olympus 765 UZ digital camera. The nominal field of view of the camera was 43° by 38°. This resulted in an area of about 8 acres with 1.05 ft resolution. Images were processed using ENVI version 4.3 (RSI, Boulder, CO) (Fig. 1B). The actual sampling area was selected in each field image resulting in four images per field. Within each image, pixels were classified using unsupervised classification with three classes. The classes represent healthy beets, diseased beets and background (soil). An area and percent of each class was calculated for each image. Statistical analysis was done on each classified image to determine the spatial distribution of diseased plants in the sampled area.

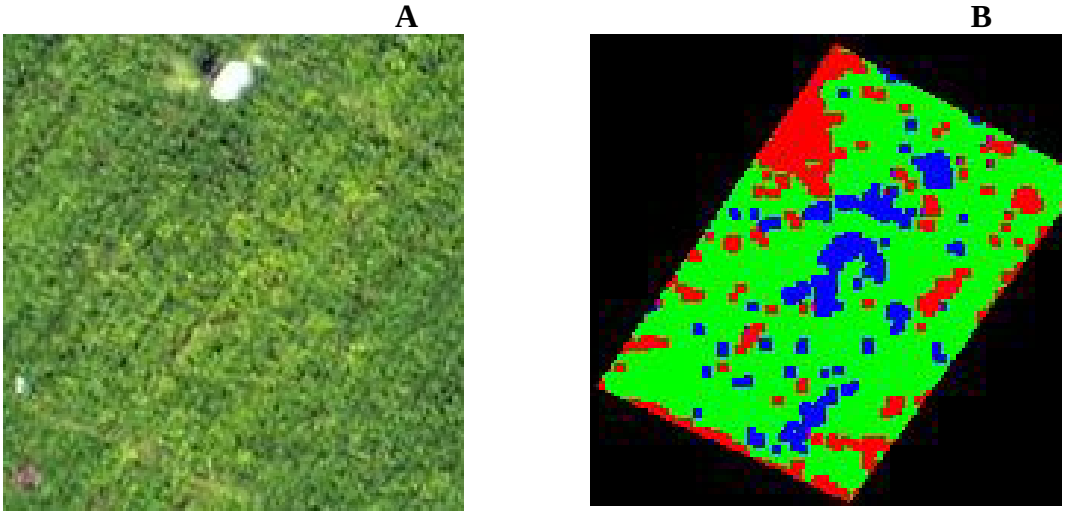

Figure { SEQ Figure * ARABIC }. Aerial image of sugar beet sampling area. Image on left is original image and image on right
is classification where light areas = healthy beets, and dark areas = blinkers. The blinkers developed in an aggregated pattern.

Soil Characteristics. Fields with discrete patches of diseased sugar beets were selected for this study. At each sampling location in each field, four soil samples were taken inside and four outside of the disease patch. Each individual sample was a composite of four, 1" diameter cores taken to a depth of 1'. Soil cores were dried, ground, and sent to Servi-Tech Laboratories for a complete chemical and physical analysis. Paired t-test analysis was conducted to determine if any of the measured variables from samples taken inside and outside of disease patches were significantly different.

Soil Inoculum Density. Currently, the only way to quantify inoculum density of BNYVV in the soil is to conduct the most probably number assay, which is a very time consuming and inaccurate procedure. For this reason, we attempted to develop a molecular technique that would detect and quantify BNYVV directly from the soil. A soil dilution series with varying amounts of BNYVV-infested rhizosphere soil was made and used in these studies. Rhizosphere soil is essentially the same as tare soil, i.e., that which remains attached to the collected sugar beet roots. The rhizosphere soil we used came from severely infected sugar beets that possessed extremely "hairy" roots and therefore, it contained a very high proportion of decayed infected root material and sporosori of *Polymyxa betae,* the soil fungus that vectors BNYVV. Initially, only the undiluted rhizosphere soil was used. Rhizosphere soil was pulverized using a bead beater and total RNA was extracted using an RNA extraction kit and following manufacturer's instructions. The total RNA was then used in a real time quantitative PCR assay to test for the presence and quantity of viral RNA using primers and probes specific for BNYVV RNA2.

Emergence of New Resistance-Breaking Strains of BNYVV. This study was a continuation from 2005 and the same methods were used. Symptomatic and asymptomatic plants were taken to the TAES plant pathology lab in Amarillo and total RNA was extracted from all plants. Extracted RNA was used to generate cDNA, which in turn was used as template for PCR amplification. Specific primers for RNA 3, the RNA species which has been associated with symptom expression and disease severity, were used to amplify the entire P25 ORF on RNA 3. DNA bands of the expected size were generated. The DNA bands were excised from the electrophoresis gel and these were gel purified and sent off for sequencing. Sequence data was analyzed using a variety of DNA analysis software programs, to determine whether differences between wild type and resistance breaking isolates could be identified.

RESULTS AND DISCUSSION

Seed Purity. Results of the blinker and spatial analysis studies were similar to those from previous years and supported our conclusion that incidence and severity of rhizomania in fields planted to resistant cultivars is not a result of seed purity or seed production issues. In the blinker study, healthy, asymptomatic plants had a significantly greater percentage of plants that possessed the *Rz* gene than those in the blinker group. Healthy plants also had significantly higher root weight and percent sucrose and a lower disease rating than the blinkers (Table 1). More importantly however, when only the blinkers were analyzed, 81% tested positive for the *Rz* gene. Furthermore, there was no difference in disease rating, the number of blinkers that tested positive by ELISA for BNYVV, or the average BNYVV value (virus concentration in infected plant tissue) between blinkers that possessed the *Rz* gene and those that didn't (Table 2). This means that without question the *Rz* gene was overcome by BNYVV.

Table 1. Disease rating and yield data from all samples[x]

Symptom	Percent *Rz*	Disease Rating[z]	Mean Root Wt.(lbs)	Sucrose (%)
Healthy	98*	0.5*	2.04*	15.84*
Blinker[y]	81	2.5	0.96	14.47

[x] Healthy means followed by an asterisk are significantly different from blinker means.

[y] Blinker is the term used to describe an individual sugar beet infected by BNYVV, which exhibits the fluorescent yellow foliage typically associated with rhizomania, surrounded by healthy beets with dark green foliage.

[z] Severity of rhizomania was based on a 0 – 4 scale, where 0 = healthy disease free roots and 4 = severe stunting, root constriction, and massive root proliferation.

Table 2. Results for Blankers only[x]

Rz Category	Percent in Category[y]	Disease Rating[z]	BNYVV Positive	BNYVV Value
***Rz* Positive**	81	2.5 NS	94NS	1.0NS
***Rz* Negative**	19	2.8	97	1.1

[x] Blinker is the term used to describe an individual sugar beet infected by BNYVV, which exhibits the fluorescent yellow foliage typically associated with rhizomania, surrounded by healthy beets with dark green foliage.
[y] Means in the top row followed by an asterisk are significantly different from those in the second row.
[z] Severity of rhizomania was based on a 0 – 4 scale, where 0 = healthy disease free roots and 4 = severe stunting, root constriction, and massive root proliferation.

An interesting aspect of the blinker study (data not shown) became apparent when data from the American Crystal area was compared to results from the Southern Minn area. In healthy beets, sucrose was higher in those from the Crystal area but this could have been due to the fact that fields in the Crystal area were sampled two weeks later in the season than those in the Southern Minn area. However, when only the blinkers were evaluated, the mean disease rating was higher in beets from the American Crystal area and mean sugars were not significantly different. This result suggests that disease was more severe in the American Crystal area and losses were greater.

A second interesting result of the blinker study was observed when a subset of data from a single field in Southern Minnesota planted to Beta 4811 was analyzed. Beta 4811 has displayed exceptionally strong resistance to rhizomania and is widely planted in the southern production area. Although no discrete spots of rhizomania existed in this field, one end of the field exhibited an exceptionally large number of blinkers. These were sampled and it was quickly realized that some of the blinkers had large, perfectly formed roots with no symptoms of rhizomania while others were severely infected and displayed typical symptoms of rhizomania. When only the blinkers were analyzed, those possessing the *Rz* gene had significantly lower disease ratings than those without the *Rz* gene. Furthermore, when the total blinkers were divided between those with severe root symptoms and those without, there were several interesting differences between the two groups of plants. Those blinkers without root symptoms had a significantly higher incidence of the *Rz* gene, significantly lower disease severity and significantly higher sucrose content and root weight. These results suggest that the virus population in this field is in the midst of an evolutionary shift. The genetics of 4811 are such that resistance is still active against most BNYVV in the field but some isolates may be beginning to develop the ability to break that resistance. The isolates of BNYVV obtained from the blinkers with the *Rz* gene, both those with and without severe root symptoms, will be highly valuable for future study and further molecular analysis.

A third interesting aspect of the Blinker study had to do with the impact of nitrate nitrogen on disease and sucrose content. In every sample that was tested, except two, healthy, asymptomatic beets always had higher sucrose content than blinkers in paired tests. However, in the two exceptions where the healthy beet sample had a lower sucrose content, the ppm of nitrate in the samples exceeded 130 ppm in one and 200 ppm in the other. In one of these, the disease rating of the blinker sample was 2.8 and the sucrose content 14.4, while in the paired healthy sample the disease rating was 0.5 but the sucrose content was only 11.8. The exact same trend was observed in the other sample. These results demonstrate the extreme importance of nitrogen management, even in fields with high disease pressure. In most cases, ppm nitrate was significantly higher in healthy than in blinker samples and this may partially explain the fluorescent yellow coloration of BNYVV infected plants. However, dispite higher ppm of nitrate in the healthy plants, proper nitrogen management allowed high root yields and high

sucrose contents. It was only when excessive nitrogen was present that sucrose content was severely reduced and nitrogen caused a greater loss in sucrose than disease.

In the spatial distribution study, the spatial patterns of the pixels were statistically determined to ascertain whether they follow a random or aggregated pattern. In all fields, the distribution of the pixels followed an aggregated pattern (Fig. 1B), with spatial autocorrelations ranging between 0.54 and 0.81 on the scale where 0 represents a random distribution and 1 represents a strongly aggregated distribution. Plant disease, resulting from a mixture of susceptible and resistant seeds, or plants with and without the *Rz* gene would display a uniform or random pattern in the field, and would not be distributed in aggregated patterns. Aggregated stress patterns usually arise from soil inhabiting infectious agents such as fungi, bacteria or viruses and are restricted in movement or due to localized soil chemical constituents. The results of this study verified statistically what is visually obvious even from ground level, i.e., disease is occurring in clusters and this spatial pattern could not reasonably occur from planting mixed seed or seed that lacked the *Rz* gene due to problems during hybrid seed production. The results from these two studies support the hypothesis that resistance breaking isolates of BNYVV have emerged and are causing rhizomania in Minnesota.

Soil Characteristics. Analysis of soil chemical and physical characteristics from inside and outside discrete patches of plants exhibiting severe symptoms of rhizomania revealed no significant differences. In this study, samples were taken only to the depth of one foot and it is possible that differences may have been found in the lower soil horizons. However, result of this study do not support the idea that rhizomania in fields planted to rhizomania resistant cultivars is due to variability in the soil.

Soil Inoculum Density. Our attempts to develop a molecular test to directly detect and quantify BNYVV directly from the soil were unsuccessful. Rhizosphere soil for this study was obtained in late September and to date we have only conducted a single round of experiments. It is recognized that soils often have properties that interfere with extraction of RNA, especially soils with high organic matter. Although initial tests were unsuccessful, we believe the technology exists to successfully extract and quantify BNYVV RNA from the soil but it will take further experimentation to identify the factors that interfere with the extraction and amplification process.

Emergence of New Resistance-Breaking Strains of BNYVV. In this study, isolates of BNYVV were obtained from fields in both Minnesota and California. In resistance breaking isolates from California we were able to detect a specific unique amino acid motif, VLE, that distinguished these isolates from wild type isolates. Isolates of wild type BNYVV that were unable to cause rhizomania in infected resistant plants did not possess the VLE motif but rather displayed an ALD or ACD motif. Using a specific application of real time PCR termed allelic discrimination, we were able to use the amino acid motif as a marker to identify resistance breaking isolates of BNYVV without the time and expense of full length sequencing. Unfortunately, resistance breaking isolates from Minnesota did not possess this marker and we are still unable to distinguish those using molecular tests.

Virus isolates baited from soil samples collected from rhizomania patches and surrounding asymptomatic areas in the field were genotyped using real-time allelic discrimination assays. Most of the isolates (11 out of 13) baited from the diseased areas carried

the resistant breaking VLE motif. By contrast, just two out of 22 isolates collected from the surrounding green areas were resistance breaking isolates and the rest were wild type strains. The near exclusive presence of resistance breaking isolates of BNYVV in rhizosphere soil from rhizomania patches suggests that they have gained a fitness advantage over wild type isolates, under the specific host-environment (*Rz1* cultivars) to which they had been exposed in the field. Also, the occurrence of mixed infections (resistance breaking and wild type) revealed that sometimes, during development of rhizomania in the field, wild type and resistance breaking isolates can coexist in the same *Rz1*-plant. However, this condition is apparently very unstable.

The almost complete exclusion of wild type isolates of BNYVV from the rhizomania patches suggests that over time resistance breaking isolates likely will become the dominant stain in the field. Therefore, new sources of resistance to BNYVV, other than *Rz1*, need to be incorporated into regionally adapted cultivars in order to maintain a viable sugar beet industry. However, in order to insure long term effectiveness of any genetic resistance, it is imperative to elucidate the mechanisms involved in resistance breakdown because incorporation of new dominant resistance genes will be exposed to the same selection pressures as was *Rz1*. The fact that we have identified on numerous occasions, severely diseased plants in fields planted to a cultivar with the *Rz2* resistance gene supports this contention.