## Studies on Detection and Intra-Plant Spread of Resistance-Breaking BNYVV

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Over the last few years, there has been much speculation as to whether inoculum density of *Polymyxa betae*, the vector for BNYVV, affected development of Rhizomania. Using traditional methods, detection of BNYVV and its vector in infested fields is a time consuming task.  Field soil is planted with seeds of a susceptible sugar beet variety and the resulting bait plants are grown for 8 - 12 weeks and then tested by Enzyme-linked Immunosorbent Assay (ELISA) for presence of the target virus or vector. Also, there has been considerable debate concerning resistance breaking (RB) isolates of BNYVV in Minnesota/North Dakota production areas.  Research on resistance breaking isolates of BNYVV is a complex and expensive endeavor. However, in our estimation, determining how/why RB isolates of BNYVV evolve and whether they will become dominant in infested fields is crucial in breeding efforts to develop cultivars with durable resistance to Rhizomania.  Therefore, a series of studies were initiated to develop a faster method of identifying viruliferous *P. betae* in field soils, to determine if inoculum density of *P. betae* was associated with appearance of rhizomania in resistant cultivars, and to determine how different resistance genes impact mutation in BNYVV.

## Materials and Methods

*Impact of P. betae inoculum density on disease severity.* In a preliminary study, infested soil was collected from a sugar beet field known to contain viruliferous *P. betae*.  Infested soil was combined with non-infested soil to create a dilution series of 1:10, 1:100, 1:1000, and 1:10,000 (w:w) soil treatments.  Five seeds of a susceptible sugar beet cultivar (AC 725) were planted in pots containing 50g of each soil dilution.  After emergence, seedlings were thinned to 3 per pot and after approximately 10 weeks plants were harvested.  Total RNA was extracted from roots using the RNeasy Plant Mini Kit.   After RNA extraction, samples were quantified and tested by Relative Quantification Real-time PCR.   Real-time PCR was conducted using TaqMan One-Step RT-PCR.   Relative quantification was used to reduce error between samples during extraction procedures.  Relative quantification uses both a primer and probe set specific for the target sequence, in this case BNYVV and the 18s Ribosomal sequence within each sample.  An endogenous 18s ribosomal control was used to normalize quantification of an internal RNA target (BNYVV sequence) for differences in the amount of total RNA added to each reaction, thereby limiting variation among samples.  Results were displayed for each individual sample by determining the virus content of a sample as 10X greater than that of a calibrator sample which contains a low target virus content.

Following the preliminary study, soil was collected from inside and outside of Rhizomania "disease spots" from several fields planted to a Rhizomania resistant variety.  Soils were potted and seed of AC 725 were planted.  After 12 weeks, plants were harvested and tested by quantitative real time PCR (described above) to determine whether virus titer was different for plants grown in soils from inside or outside of Rhizomania spots.

*Development of rapid detection method for BNYVV in infested soils.*  Infested rhizosphere soil was collected from three sugar beet varieties, from a variety trial in Crookston MN.  Samples were collected from a Vanderhave Tandom Technology variety, which showed the greatest resistance to Rhizomania, Beta 46519 with strong resistance, and AC 725 a susceptible variety.  Half the soil from the susceptible plants was air dried, while the other half, and soil samples from the other varieties, were sealed in a zip-lock bag to keep the soil moist.  The moist soil from the Tandom Technology variety, Beta 46519, and the susceptible variety were used as 3 individual treatments.  Dried soil from the susceptible was divided into 100g subsamples, in three separate beakers, and augmented with .5g, 1.0g, and 1.5g of dried roots infected with BNYVV, thereby creating three more soil treatments, for a total of 6 treatments.  Four replicate Petri plates were filled with 25g of each soil and 75 ml of sterile distilled water was added to each plate.  Susceptible sugar beet seedlings were then floated in each Petri dish for 24 hr, 4 days, or 7 days.  After 24 hours, seedlings from two of the four replicates were harvested, the third rep was harvested at 4 days and the 4th rep was harvested after 7 days of incubation.  Roots of three plants within one treatment were separated from their stems and combined as one composite sample.  The roots were then washed with distilled water to remove any remaining soil and frozen at -80C until RNA extraction and detection by Real-time PCR could be performed.  RNA extraction was conducted as described below.

*Characterization and dynamics of zoosporic inoculum production.* As an initial step to characterize the dynamics of zoosporic inoculum production, a lab protocol was developed to isolate zoospores from liquid media. Roots of susceptible and *Rz2*-resistant plants, grown in soil infested with viruliferous or non-viruliferous *P betae*, were harvested 4-6 weeks after seeded. The infectious inoculum consisted of wild type BNYVV previously propagated

during three host-passages through susceptible or *Rz2*-plants. These two virus lineages were referred as W or B, respectively. Thus, the treatments were W-inoculum in susceptible (W/S) or *Rz2*-plants (W/R), B-inoculum in susceptible (B/S) or *Rz2*-plants (B/R), and non-viruliferous in susceptible plants (NV). Four to five plants were soil-inoculated by treatment. Individual plants were analyzed by realtime RT-PCR for virus content, and composite samples of 0.5 g of dry root tissue were prepared for zoospore isolation. The release of zoospores was induced by adding 5 ml of distillated water to the composite root samples.

*Impact of host resistance genotype on mutation in BNYVV.* Three sugar beet varieties, a susceptible check, one with *Rz1* resistance, and one with *Rz2* resistance, were used in this test. A sterilized potting soil was infested with a well characterized isolate of BNYVV and plants of each variety were then grown in the infested soil. After approximately 12 weeks, plants were harvested, total RNA extracted, and traditional PCR performed. BNYVV amplicons from the PCR were cut from the gel, purified and cloned. Clones were then sequenced and analyses were performed to quantify the amount and type of mutation in clones of BNYVV isolated from the different host plants.

## Results and Discussion

*Impact of P. betae inoculum density on disease severity.* Results from this study demonstrated a positive correlation between soil inoculum density and virus titer in plants growing in the different soil treatments (Fig. 1). These findings verify that higher inoculum densities do result in more severe disease, as evidenced by higher virus titers. They also suggest that differences in inoculum densities from soils inside and outside of rhizomania spots should be detectable, if the rhizomania spots are a result of higher inoculum density. Although the methods used in this study are valid, a more rapid and precise method for determination of the number of infectious *P. betae* sporosori in the soil is needed.
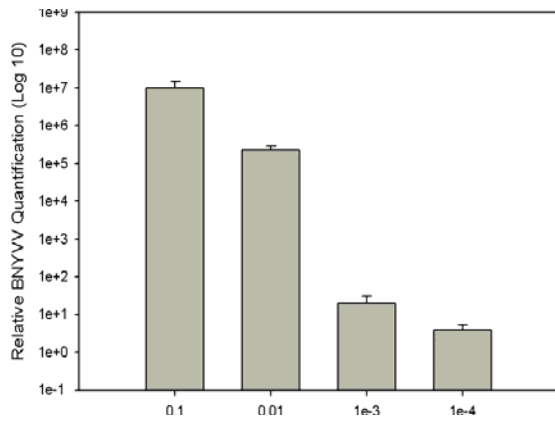


**Fig. 1**. Relation between inoculum density and virus titer.

*Development of rapid detection method for BNYVV in infected soils.* Beet necrotic yellow vein virus was detected in all treatments amended with infected roots as well as the susceptible control soil that remained wet after 24 hrs, 4 days and the 7 day period. Inexplicably, the susceptible control dry soil, amended with .5g of roots, tested negative after 7 days. Soil from one replication of the Beta 46519 treatment tested positive for BNYVV after 24 hrs, but the other replication was negative. All Tandom soil treatments tested negative for BNYVV at all incubation periods. This study demonstrated that BNYVV can be detected from bait plants after 24 hr incubation in an infested field soil solution. This method needs to be fine tuned but it has potential to greatly reduce the time and expense to test soil samples for the presence of viruliferous *P. betae*.

*Characterization and dynamics of zoosporic inoculum production* Motile cells were abundant after 2-3 h at room temperature and remained viable up to 2 days in liquid. Zoospores contained in 3 ml of recovered liquid were concentrated in 50-100 μL by centrifugation at 4000 g by 5 min after removal of plant debris by filtration. Non-concentrated zoospore suspensions from each treatment were also used as inocula. Susceptible and *Rz2*-plantlets, germinated in a sterile substrate (Agrolite), were immersed by 2 h in the same zoospore suspension in parallel. Extraction of total RNA from plant and zoospores was performed using the RNeasy kit (Qiagen Inc.), but zoospores first were disrupted by vortexing in 10 mM TRIS plus 0.5% SDS, pH 8.0.
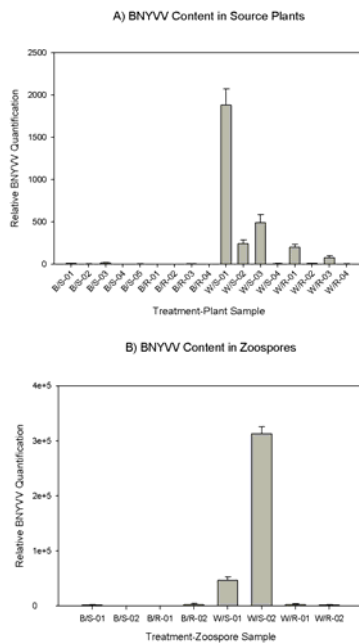


**Fig. 2**. Content of BNYVV detected by realtime RT-PCR in susceptible (S) and Rz2-resistant (R) source plants (A) and zoospores of *P. betae* (B).

The content of BNYVV in sugarbeet plants used as source of zoospores was affected by the original source of the inocula and the genotype of the infected plant from which zoospores were collected. Thus, wild type BNYVV that was propagated in resistant *Rz2*-plants caused mild virus infections (low virus titer) in both susceptible and resistant plants. By contrast, the same strain maintained in susceptible plants caused severe virus infections in susceptible plants and moderate infections in Rz2-plants (Fig.2A). The virus content in the source plants paralleled the virus content in zoospores released from those plants (Fig. 2B). The low virus titer in the B/S treatment could be explained by a loss of virus fitness in susceptible plants after passage through resistant plants, or by a reduced amount of initial inocula generated in the rhizosphere of resistant plants. In fact, previous assays indicate that inoculum density in the soil is directly correlated with BNYVV titer in the bait plants (Fig. 1).

DNA sequencing of viral RNA from zoospores was possible only from samples where significant amount of viral RNA was detectable by PCR. Preliminary data revealed that the predominant haplotypes present in the source plants were also present in the corresponding zoopspore populations. However, we have not yet determined whether mutant haplotypes of BNYVV that are acquired by zoospores from resistant plants are biologically fit and can be transmitted to another plant.

*Impact of host resistance genotype on mutation in BNYVV.* As expected, virus titer in the different varieties was directly proportional to strength of resistance, so titer was highest in the susceptible control, intermediate in the *Rz1* variety, and lowest in the *Rz2* variety. In total, 385 cDNA fragments, representing 26 single-plant populations (isolates), were sequenced in both directions. The number of clones, mutation frequency ($\mu$), number of segregating sites per nucleotide site [$\theta(S)$], and nucleotide diversity ($\pi$) per host genotype and serial passage was determined (Table 1). The overall nucleotide diversity ($\pi$) detected in host-passage populations (*i.e.*, a population composed of all isolates that belong to the same host passage and plant genotype, for instance, *1Rz1* population, was higher in resistant than in susceptible plants ($P = 0.07$). The intraplant viral $\pi$ was statistically significant only between plant genotypes of the second host passage. However, most of the differences between plant genotypes were explained by the interplant virus diversity, which was up to ten times higher in the most resistant *Rz2*-plants than in the susceptible control. The results of this study suggest that host genotype affected the genetic diversity of BNYVV by the amount and frequency of different mutations in the population, and that mutation was significantly and positively correlated to strength of genetic resistance.

**TABLE 1**. Host effect on the genetic diversity of BNYVV populations during the first and second serial host passage.

| Serial Passage | R-gene | Clones/Isolates | $\mu$ [a] | $\theta$ (S) [b] | $\pi \pm SE \times 10^{-3}$ [c] | | |
|---|---|---|---|---|---|---|---|
| | | | | | Intraplant | Interplant | Total |
| 1 | *Rz1* | 32/4 | 14/31,168 | 3.23 ± 1.3 | 0.98 ± 0.3 | 0.51 ± 0.3 | 1.49 ± 0.5 a |
| | *Rz2* | 40/4 | 12/38,960 | 3.29 ± 1.3 | 0.63 ± 0.2 | 0.83 ± 0.5 | 1.46 ± 0.6 a |
| | S | 42/4 | 9/40,908 | 1.86 ± 0.8 | 0.57 ± 0.2 | 0.12 ± 0.1 | 0.69 ± 0.3 b |
| 2 | *Rz1* | 80/5 | 41/113,040 | 8.08 ± 2.4 | 0.85 ± 0.1 | 0.15 ± 0.1 | 1.00 ± 0.2 a |
| | *Rz2* | 114/5 | 87/161,082 | 16.20 ± 4.2 | 1.25 ± 0.1 | 0.63 ± 0.4 | 1.88 ± 0.4 a |
| | S | 77/4 | 27/108,801 | 5.49 ± 1.7 | 0.61 ± 0.1 | 0.02 ± 0.03 | 0.63 ± 0.1 b |

[a] Number of mutations (nucleotide substitutions) over total sequenced nucleotides of the p25 (RNA 3) region. The sequenced fragment was 974 and 1413 nucleotides long for the first and second host passage, respectively.
[b] Number of segregating sites per nucleotide site and standard deviation estimated by the Kimura 2-parameter (Schneider *et al*., 2000).
[c] Nucleotide diversity estimated by the Kimura 2-parameter model as implemented in MEGA 3.1 software (Kumar *et al*., 2004), and SE estimated by 500 replicates bootstrapping. Calculations corroborated using DnaSP v4.10.7 software (Rozas *et al*., 2003). Total $\pi$ values with the same letter were statistically identical (Tukey HSD Test, $P = 0.07$).