

METABOLOMIC ANALYSIS FOR IDENTIFICATION OF BIOLOGICAL FUNCTIONS ASSOCIATED WITH INFECTION BY AND RESISTANCE TO *BET* NECROTIC YELLOW VEIN VIRUS IN SUGARBEET

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Background:

Rhizomania, caused by *Beet necrotic yellow vein virus* (BNYVV), is one of the most economically important diseases affecting sugarbeet, and is widely distributed in most sugarbeet growing areas of the world. Fields remain infested with BNYVV indefinitely in *P. betae* cystosori that can remain dormant up to 25 years. Rotation to non-host crops or lengthening rotations is ineffective at reducing disease incidence, and to date the only viable means of control has been natural host-plant resistance. The *Rz1* source of resistance was introduced widely to commercial sugarbeet in the 1990s, and for several years has effectively controlled the virus. However, this resistance does not completely eliminate virus replication, but rather suppresses it to low levels compared to what one would find in a susceptible variety. The low level of replication in *Rz1* sugarbeet, has led to the emergence of new variants that overcome or “break” the resistance, since the main forms of the virus that can replicate in *Rz1* varieties are those few variants that have the ability to replicate in the presence of *Rz1*. In the early 2000s an *Rz1* resistance-breaking variant emerged in the Imperial Valley of California (Liu et al., 2005; Rush et al., 2006), and subsequent studies identified the presence of limited numbers of isolated resistance-breaking (RB) variants from most American production regions (Liu and Lewellen, 2007). RB isolates are increasingly affecting production throughout the US industry, and this can be expected to continue.

In addition to the well-known *Rz1* gene, several additional sources of genetic resistance to BNYVV have been identified, and they hold promise. Although these additional genes are being incorporated into sugarbeet varieties, the inability of any of these genes to completely eliminate BNYVV replication leaves all known resistance genes prone to eventual breakdown, even when the genes are “stacked” or combined in order to enhance resistance and make it more difficult for RB strains to establish in plants. It is critically important that rhizomania be studied to allow it to remain under control, and new advances in research approaches create the opportunity for completely new strategies for control of pathogens. This proposal uses one of those new approaches, metabolome analysis, to enhance knowledge of what happens in sugarbeet during infection, as well as to learn how resistance changes the sugarbeet plant to reduce virus accumulation and prevent symptom development. The result of this project will clarify information gained using other technologies and lead to new strategies to enhance and stabilize known forms of resistance. The information generated should also lead to new screening methods that can be applied for identification of varieties with enhanced resistance, as well as for the identification of new approaches to protect sugarbeet from rhizomania.

The different sources of resistance to BNYVV map to different chromosomal positions and although some may be allelic to one other, others appear to be distinct (Scholten et al., 1997; 1999; Gidner et al., 2005). Furthermore, several minor genes may contribute to enhanced resistance (Gidner et al., 2005), although further characterization of how this works is necessary. With the introduction of Roundup-Ready sugarbeets a few years ago, we may begin to see new opportunities for the application of biotechnology-based resistance at least in the US and Canada. Furthermore, the sequencing of the sugarbeet genome and the aggressive development of genetic markers creates additional opportunities for selective breeding that can target development and selection of sugarbeet with specific and unique traits that may lead to enhanced resistance as we learn more about how BNYVV infects sugarbeet and overcomes known sources of resistance. Finally, the emergence of new gene editing technologies may also lead to the ability to specifically target individual genes for up-or down-regulation that may enhance pathogen resistance or yield related traits.

With these things in mind, we recently completed studies evaluating resistant and susceptible sugarbeet using proteomics methods in order to gain an understanding of what BNYVV does at the cellular level to allow it to infect and cause disease in sugarbeet. These studies led to new knowledge on how BNYVV infection and virus resistance alter protein expression associated with infection and resistance in sugarbeet. (Larson et al., 2008; Webb et al., 2014 & 2015). Others have used different strategies to identify other protein interactions that may contribute to infection

(Thiel and Varrelmann, 2009). Recent studies have begun to examine how gene activity (transcriptomics) is influenced by BNYVV infection (Fan et al., 2014; 2015). All of this information is informative on its own, but studies on gene expression (RNA and protein) only provide part of the picture. By utilizing a metabolomics approach, we can complement knowledge of gene expression provided through previous studies, and obtain a more complete picture of what is happening during BNYVV infection and resistance.

Metabolomics provides an analytic tool that enables the qualitative and quantitative profiling of metabolites in a biological system and serves as a link between the plant genotype and phenotypic (visible or obvious) responses (Fiehn et al., 2000; Heuberger et al., 2014). A better understanding of plant metabolism in response to different biotic stresses (including pathogen infections) facilitates a better understanding of plant physiology which is crucial for the development of future applications in plant breeding, biotechnology, and crop protection (Aliferis and Jabaji, 2012a). Metabolomic profiling was previously used in sugar beet to complement proteomic studies characterizing the response of carbohydrate metabolism and the TCA cycle to iron deficiency (Rellan-Alvarez et al., 2010). Aliferis and Jabaji (2012b) have described the metabolome of potato in response to infection with *R. solani* AG 3; however, to date there have been no known reported studies on the effects of plant pathogens on the metabolome of sugar beet. Additionally, metabolomic approaches have been utilized in characterizing compounds found in *R. solani* that are directly important to the fungus during critical life stages as well as characterizing the metabolites found in fungal exudates which may contain clues to secreted effectors (Aliferis and Jabaji, 2010a,b). Application of this technology to BNYVV in sugarbeet should lead to the identification of compounds necessary for both infection and resistance.

The information provided through our studies have the potential to lead to a new era in management of BNYVV. By utilizing knowledge of how the virus infects sugarbeet, combined with increasing knowledge of the sugarbeet genome and a growing number of molecular markers, it should be possible to enhance performance of existing resistance genes through selective, marker based breeding practices (Laurent et al., 2007). This could include selection for sugarbeet varieties enhanced for specific metabolites, as well as identifying new ways to reduce or even prevent BNYVV from infecting sugarbeet through targeted breeding directed at enhancing or manipulating specific biological pathways within the plant through specific gene editing approaches.

Objectives:

1. Compare the metabolome of a near isogenic line of susceptible (*rz1*) sugarbeet with those of sugarbeet with each of two resistance genes against BNYVV (*Rz1* and *Rz2*) at specific time points in the infection process.
2. Identify important compounds/cellular chemicals that may be critical to BNYVV infection of susceptible sugarbeet and for suppression of BNYVV in resistant sugarbeet.
3. Compare results with existing knowledge of RNA and protein expression changes associated with infection and resistance from previous studies to identify targets for interference and potential resistance.

Summary of Project to Date:

Three sugarbeet lines with closely related genetic backgrounds (near isogenic lines) from the ARS-Salinas sugarbeet breeding program (developed by Lewellen and Richardson) were used in seedling grow-out experiments; one with the *Rz1* resistance gene (C79-1), another with the *Rz2* resistance gene (C79-3), and one susceptible to BNYVV (C37). All three lines share the same genetic background and differ by the type of *Rz* gene they carry. Soil containing a well established isolate of BNYVV pathotype A (source collected from USDA-ARS, Spence Field in Salinas in 2006) was mixed with equal parts sterile builders sand and placed into new Styrofoam cups. Parallel studies were performed with an *Rz1* resistance breaking strain of BNYVV (Imperial Valley, CA - Rockwood 158 RB isolate), as well as with virus-free (healthy) soil. For each sugar beet variety, 50 seeds were planted per cup, with two cups per treatment, and grown in a growth chamber at 24°C with 16-hour days and approximately 220 μM $\text{m}^{-1}\text{s}^{-2}$ light until 3 weeks after sowing. At each time point, seedlings from each cup was handled independently for each treatment to assure good infection of each sample/treatment. Foliar and root portions of the plant were

separated at the crown and lyophilized (freeze dried), then stored at -80 until further analysis. Root samples from each plot were tested by RT-PCR to confirm BNYVV infection prior to use in metabolome analysis, and remaining roots from the same samples were used for metabolite extractions.

Roots from all three experiments were freeze-dried and stored at -80C so that metabolites could be extracted from all samples at the same time. Upon completion of the last replication, dried root samples were pulverized in liquid nitrogen and sent to the Core Laboratory at Colorado State University (CSU) in Ft. Collins, CO for methanol extraction of metabolites. Metabolome analysis was completed at CSU during the fall, and results of analyses provided to USDA-ARS in late November 2017.

Overall 746 metabolites were found and these were annotated to known compounds or to unknown compounds with a specified mass. These metabolites were examined in all possible combinations of treatments to look for statistically different levels of expression among treatments, including patterns of expression indicating how traditional or RB-BNYVV influence resistant and susceptible sugarbeet during infection, as well as for identification of “interesting” compounds that may play an important role in rhizomania disease development. Metabolite levels were compared among treatments using a 95 percent confidence interval to distinguish compounds with statistically different levels of expression among treatments. Results demonstrated the most important difference in metabolite levels was between healthy sugarbeet plants and sugarbeet plants infected with BNYVV. Results also demonstrated differences between traditional BNYVV and RB-BNYVV. Overall, comparative studies indicated 32% of differences in metabolite levels among treatments were based on the presence or absence of BNYVV (Fig. 1). In contrast, only 3% of variation among treatments could be explained by differences in sugarbeet variety (i.e. the different resistance genes) (Fig. 2). Essentially, results indicate most metabolic differences are caused by the BNYVV infection, and are not influenced much by the presence or absence of either resistance gene. This contrasts with what was observed with our recent proteomics analysis of similar sugarbeet near isogenic lines, in which differences that occurred were influenced by both virus strain and the resistance genes.

In our previous proteomics analysis comparing BNYVV infection of Rz1 and Rz2 sugarbeet with susceptible sugarbeet, we identified a number of proteins with differential expression not only between RB- and traditional strains of BNYVV, but also between sugarbeet genotypes (Rz1, Rz2, and susceptible). Results of those studies demonstrated that abundance of select proteins in sugarbeet is significantly altered based on the presence or absence of the two resistance genes (Webb et al., 2015), whereas in the current metabolomics study very limited (3%) differences in the metabolome were determined by the presence or absence of rhizomania resistance genes.

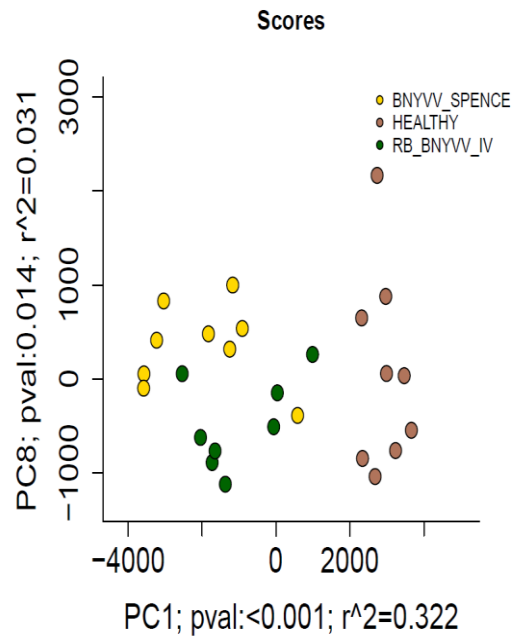


Figure 1. Principle component analysis plot generated from 27 samples derived from 9 treatments showing clear separation by virus type. Yellow: BNYVV-Spence (traditional/wild type BNYVV), Green: BNYVV-IV (Rz1 Resistance breaking BNYVV), Red: Healthy (virus-free sugarbeet).

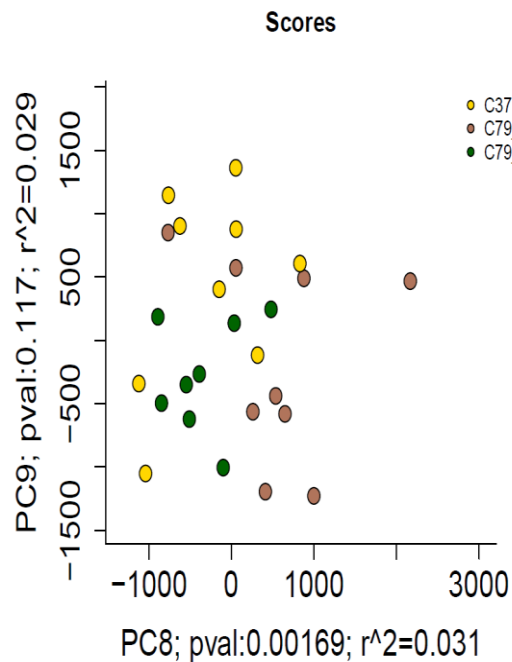


Figure 2. Principle component analysis plot generated from 27 samples derived from 9 treatments showing little separation by sugarbeet genotype (resistance gene or not). Yellow: C37 (susceptible sugarbeet [rz1rz2]), Red: C79-1 (Rz1 resistant sugarbeet [Rz1rz2]), Green: C79-3 (Rz2 resistant sugarbeet [rz1Rz2]).

Continuing studies are focusing on identification of specific compounds that differ among treatments. Although these detailed studies are just beginning, some interesting results have already been identified, including compound $C_{40}H_{107}N_{17}OS_4$ (Fig. 3). This compound had low expression in the absence of virus in both susceptible (rz1rz2) and resistant varieties (both Rz1 and Rz2), but higher expression with virus infection when either traditional or Rz1-resistance-breaking BNYVV strains were present. In general, the expression of this compound mimics what would be “expected” in a traditional gene-for-gene type of resistance. The highest expression of compound $C_{40}H_{107}N_{17}OS_4$ was observed in the susceptible line (C37) with the traditional BNYVV strain (Spence), but expression differences were also significant with the RB BNYVV strain (which we believe is generally less fit overall than traditional BNYVV based on its performance in field situations). The fact that this compound is expressed at elevated levels in all varieties indicates its expression is a response to infection, but not necessarily associated with ability of the plant to resist infection (no strong differential effect with resistant beets).

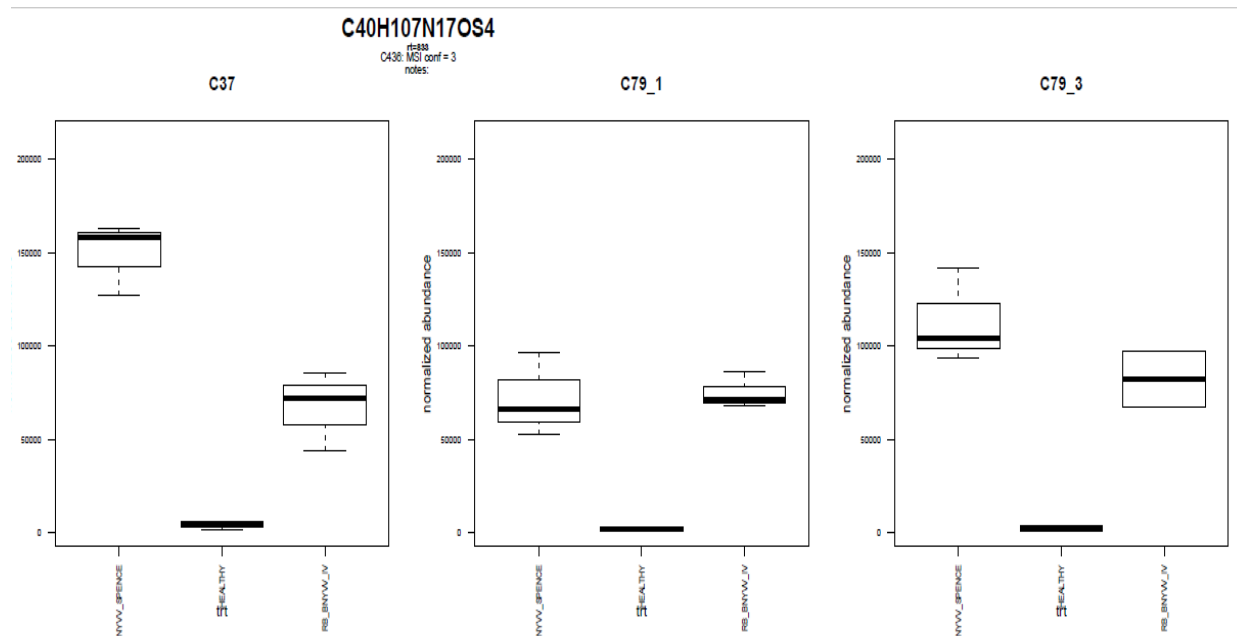


Figure 3. Abundance of compound $C_{40}H_{107}N_{17}OS_4$. C37 = susceptible sugarbeet (rz1rz2), C79-1 = Rz1 resistant sugarbeet (Rz1rz2), C79-3 = Rz2 resistant sugarbeet (rz1Rz2).

Further Research:

Although we have not requested additional funding for this project we will be continuing data analysis and interpretation of results. Through characterization of differential abundance of compounds and identification of these compounds, we expect to improve our knowledge of what is happening biochemically in sugarbeet during BNYVV infection and development of rhizomania disease. We will also examine results of this metabolome analysis in comparison to those of our previous studies on proteomics (Larson et al., 2008; Webb et al., 2014, 2015), and studies by others on gene expression and protein interactions (Fan et al., 2014, 2015; Thiel and Varrelmann, 2009). This should allow us to begin to piece together how BNYVV causes disease in plants by determining changes that occur in infected vs. healthy sugarbeet. Ultimately we anticipate gaining insight into how resistance genes are able to suppress BNYVV levels by identifying differences in biochemicals produced (this study) along with changes in gene expression (previous studies). This information will be useful toward application of marker-based selection of traits that may enhance performance of resistance genes, as well as for identification of targets for use of new biotechnology-based methods that should lead to novel methods to prevent rhizomania disease in sugarbeet.

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Budget Justification: Funds for general laboratory supplies, as well as kits and reagents necessary for metabolite extraction, and other metabolome analyses were provided through a combination of BSDF funds and USDA-ARS in-house funds (Not SBREB) during 2016. These charges were covered with 2016 funds. Plant growth work at Salinas is nearly completed, and all samples will be sent for analysis once the current and final experiment is completed this month (Dec. 2016).

A GS-11 USDA-ARS postdoctoral research associate (Dr. Navneet Kaur, ARS Salinas) will conduct data analysis, with guidance and assistance from Drs. Broekling (CSU) and Webb (USDA) in Ft. Collins. Dr. Kaur's salary for sample preparation and research on this project was provided by SBREB in 2016. We are only requesting \$6,000 from SBREB in 2017 to support Dr. Kaur's salary (additional salary funds were requested from BSDF). Dr. Kaur cannot be paid with USDA in-house funds due to her nationality (India). Therefore we are requesting limited funds from BSDF to assist with Dr. Kaur's salary to finish out the project involving data analysis and interpretation of results. An existing agreement is in place between USDA-ARS and BSDF to utilize BSDF funds for ARS salaries.

<u>Budget:</u>	<u>USDA</u>	<u>BSDF</u>	<u>SBREB</u>
Labor		\$25,000	\$10,000 \$6,000
Equipment (over \$250.00)	\$0	\$0	\$0
Supplies	\$800	\$0	\$0
Service (metabolomics analysis)	\$9,000	\$0	\$0
Travel		<u>\$0</u>	<u>\$0</u> <u>\$0</u>
TOTALS:		\$34,800	\$10,000 \$6,000