Progress Report
Research Proposal
SRSFC Project # 2016 R-04

Identification of differentially expressed genes for mummy berry (*Monilinia vaccinii-corymbosi*) disease in blueberry

Hamid Ashrafi (PI)
Assistant Professor
North Carolina State University
Department of Horticultural Science
234 Kilgore Hall, Campus Box 7609
Raleigh, NC 27695-7609
Ph: (919) 515-1216
Fax: (919) 515-2505
hamidashrafi@ncsu.edu

Marc Cubeta (Collaborator)
Professor, Soil Mycology and Plant Disease Ecology
North Carolina State University
Department of Plant Pathology / Center For Integrated Fungal Research (CIFR)
225 Partners Building III
851 Main Campus Drive,
Raleigh, NC 27695-7609
Ph: (919) 513-1227
Fax: (919) 513-0024
macubeta@ncsu.edu

Mark Ehlenfeldt, USDA-ARS (Collaborator)
P.E. Marucci Center for Blueberry & Cranberry Research
125A Lake Oswego Road
Chatsworth, NJ 08019
Phone: 609-726-1590 ex. 4421
Fax: 609-726-1593
Mark.Ehlenfeldt@ARS.USDA.GOV

Objectives

The objectives of this research are:

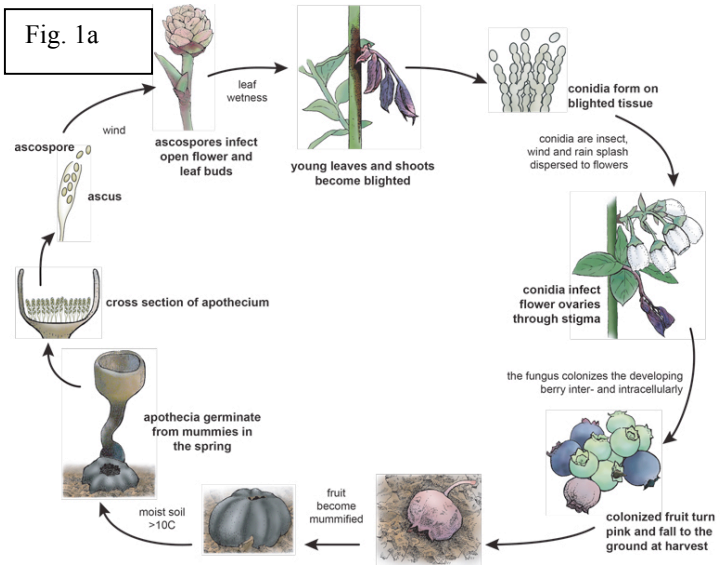
1. To generate transcriptome data using next generation sequencing (NGS) technology for infected and non-infected (healthy) blueberry plants in response to mummy berry.

2. To determine the differences in gene expression of blueberry fruits that are infected and not infected with mummy berry using expressed genes

Justification and Description:

One of the most devastating diseases of blueberry (*Vaccinium corymbosum and V. virgatum*) in the U.S. is mummy berry [1-3]. The disease was first reported in Europe in 2002 [3] and has become an increasingly significant pest in many blueberry growing regions. The causal fungus of the disease is *Monilinia vaccinii-corymbosi* (MVC hereafter). The life cycle of the pathogen is well described (Fig. 1a). The fungus overwinters on remnants of mummies from previous year(s). Cup- or spherical shape structures of the fungus (Fig. 1b), called apothecia germinate and grow in the early spring. The apothecia releases ascospores that land on and then infect shoots and flowers. Conidia produced from the primary infection are the source of secondary infection by entering into the style and ovary of the flowers. Once the disease gets established in the farm, it can damage the young shoots and flowers at the beginning of the season and later mummify the berries, which makes the fruit unusable (Fig. 1c). Complexity of the disease and long juvenility life of blueberry plant hampers the breeding efforts. We know very little about the mode of action of the pathogen and its interaction with the plant. This project provided us the preliminary data to pursue it further through future SRSFC or other grants.

Fig. 1a



Methodologies

Plant Materials: the blueberry cultivar 'Arlen' was used for transcriptome analysis in response to mummy berry disease infection. This cultivar was chosen due to its notable susceptibility to mummy berry and infected tissue availability when tissue samples were collected in 2015. It also showed sever disease susceptibility in 2017. Cultivar 'Arlen' is a southern highbush blueberry cultivar released by Dr. Jim Ballington with the USDA in 2000 as part of the NCSU blueberry breeding program [4, 5]. It was developed as a cultivar for its high fruit quality, bush architecture, self-fertility, low chill hour requirement, moderate cold hardiness, resistance to stem blight, and later ripening time [4-6]. Arlen comes from an ancestry composed primarily of northern highbush (*V. corymbosum*), with some incorporation of *V. darrowii* and *V. virgatum* (previously *V. ashei*) into its gene pool.

Tissue Collection: Tissues were collected in 2015 from various tissues of one single Arlen plant at each of the 2 different field locations, Ideal Farm Tract at Horticultural Crops Research Station in Castle Hayne, NC (CH hereinafter) and Sandhills Research Station, NC (SH hereinafter). These two locations are geographically 140 miles apart from each other. The mummified fruit was only collected from CH, because the fruit in SH were healthy and disease free. On the same tree that we found mummified fruit were able to locate a few healthy fruit. This allowed us to keep the genetic and environment variables constant in CH while the mummification was the only affected variable by mummy fruit.

The tissues immediately frozen in liquid N in order to preserve the RNA expression profile. Collection dates vary between March 20, 2015 to June 20, 2015, as they were collected at different developmental stages. These tissue samples include leaf, flowers of stages 1, 3, and 5, fruits of stages 1, 2, 3, and 4, and fully developed, mummified stage 4 fruits. Root tissue was also collected from potted bushes that were grown in the greenhouse. After collection, these samples were kept at -80°C until time for RNA extraction. RNA was extracted from 12 tissue tissues using the Sigma-Aldrich Spectrum Plant Total RNA Kit (cat #: STRN50) according to manufacturer's instruction.

RNA extraction: Each tissue was composed of multiple fruit, flower, root or leaf to increase biological replications. RNA was extracted two times from a pool of each tissue to obtain 24 RNA samples. RNA concentration was measured using a Qubit 3.0 fluorometer (Life Technologies) and the RNA broad range assay. Three readings were taken and the average of these readings were used as the concentration for each RNA sample. Quality of each RNA sample was assessed using the 2100 Bioanalyzer system (Agilent Technologies).

cDNA Library Preparation and sequencing: Illumina mRNA libraries for sequencing were prepared with KAPA Biosystems Stranded mRNA-Seq Kit for Illumina Platforms (cat #: KK8420) and the BiooScientific NEXTflex Rapid Directional RNA-Seq Kit (cat #: 5138-07). Libraries were quantified by the KAPA Biosystems Library Quantification Kit for Illumina platforms (cat #: KK4824) and the Bio-Rad CFX96 Real-Time PCR

Detection System on all samples in order to get accurate molarity concentrations for each library. Libraries were sent to Novogene Company (Sacramento, CA) for 150bp pair-end sequencing on the Illumina HiSeq 4000.

Sequence Data Analysis: Raw paired-end reads in FASTQ format were imported into CLC Genomics Workbench (v. 10.0.1, https://www.qiagenbioinformatics.com). The reads were trimmed to remove any traces of adapters, primers and polyA tail sequences. Reads shorter than 45 nucleotides were discarded, but broken pairs were saved as orphans reads. In order to remove fungal RNA sequence contamination from our blueberry reads, the trimmed reads were mapped to *M. vaccinii-corymbosi* (MVC) strain RL1 reference genome (developed in our lab). The unmapped reads were collected and used for further analysis, because these reads were hypothetically from blueberry. These unmapped reads to MVC genome were mapped to the blueberry cv. 'W85-20' reference genome.

Transcriptome Assembly: A *de novo* assembly of transcriptome was created in CLC using a word size of 31 and bubble size of 300.

Differential Expression Analysis: The Trinity RNA-Seq suite was used to perform differential expression analysis by utilizing the R packages DESeq2 and edgeR. Genes that were DE in both infected fruit replicates were collected for functional annotation. A total of 3,395 contigs were imported into Blast2GO (v. 4.1.9) and blasted using BLASTx 2.4.0+ against the nr protein database downloaded from NCBI. Blast2GO was also used for GO mapping, InterProScan, and annotation of the DE contigs. GO combined graphs and KEGG pathway maps were generated for the DE dataset.

Results

Sequencing and assembly:

We generated 1,763,299,230 short 150 base pair reads, equal to 264 Giga Base (Gb) of data. After trimming of the low quality reads and the reads that were contaminated with adapters, 1,418,513,384 reads equal to 143 Gb of nucleotides were retained. The reads were assembled with CLC Workbench (v. 10.0) software package in to 146,476 contiguous sequences (Contigs) covering 117 Mb of blueberry transcriptomes.

Differential Expression of the Genes (DEGs):

The mummified fruits collection from (CH) was compared with healthy fruits that were collected from the same location (CH) as well as those that were collected from SH collection site. This allowed us to investigate the effect of environment on differentially expressed genes as the genotypes were the same in both locations. The number of detected DEGs were in the same range across locations and analyses methods (Table 1) and it is normal to observe differences in the number of detected genes using different software packages. Overall, the results of DESeq2 and edgeR programs were highly congruent (R^2=97.6%), that is 97.6% of the genes that were deemed to be differentially expressed were detected by both analyses (Fig 1). Among all four analyses methods,

tissue types and collection sites (Table 1), a total of 2,742 genes were in common (Fig 2). These genes were functionally annotated using BLAST2GO software package. We identified 1,022 sequences that were clustered in 91 Gene Ontology terms, majority of which were associated with defense response (151), defense response to other organisms (81), response to jasmonic acid (57), ethylene-activated signaling pathway (53) and 85 more GO terms that top 10 listed in Table 2. This indicates that mummy berry is affecting the genes in these pathways and disrupts the function of the genes in the mummified fruit on opposed to not infected fruit. Jasmonic acid, ethylene-activated signaling pathway and salicylic biosynthesis pathways are well known to be involved in systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR is associated with induction of a wide range of genes known as pathogen related genes while ISR is manly dependent of the phytohormone salicylic acid (salicylate).

Table 1: Number of differentially expressed genes in ripe blueberry fruit in response to mummy berry fungus in Castle Hayne and Sandhills, NC research stations.

Number of DEGs Detected in Two Locations			
Mapping Algorithm	Differential Expression Algorithm	Number of DEGs between Mummified and Healthy Fruit in Castle Hayne	Number of DEGs between Mummified and Healthy Fruit in Castle Hayne and Sandhills
Bowtie2	DESeq2	4058	4412
	EdgeR	4370	4777

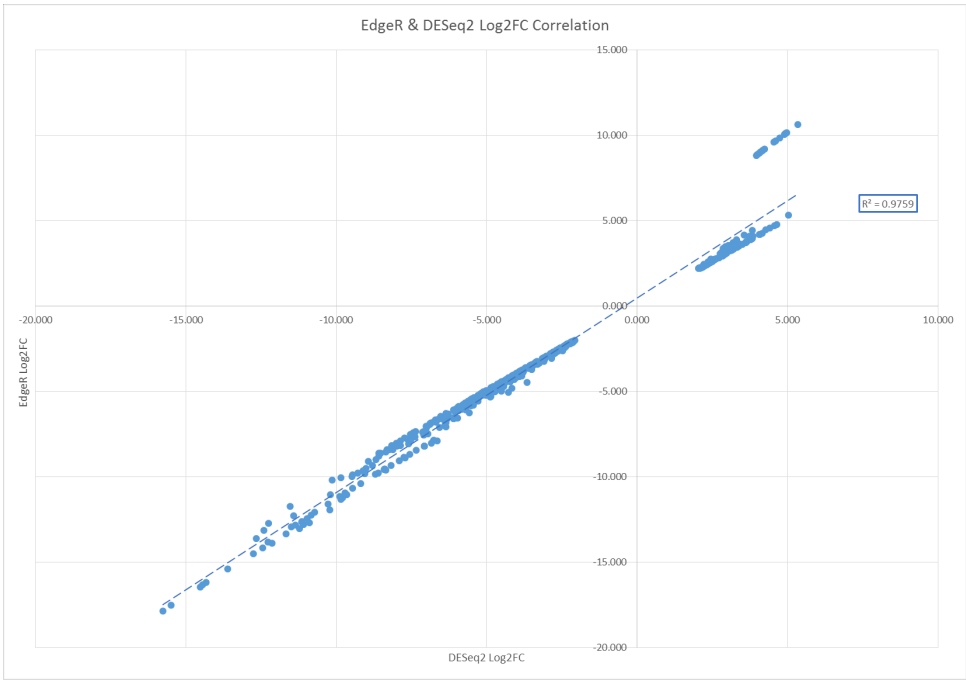


Figure 1. The correlation of the Log fold change for the genes that were identified with DESeq2 and EdgeR programs.

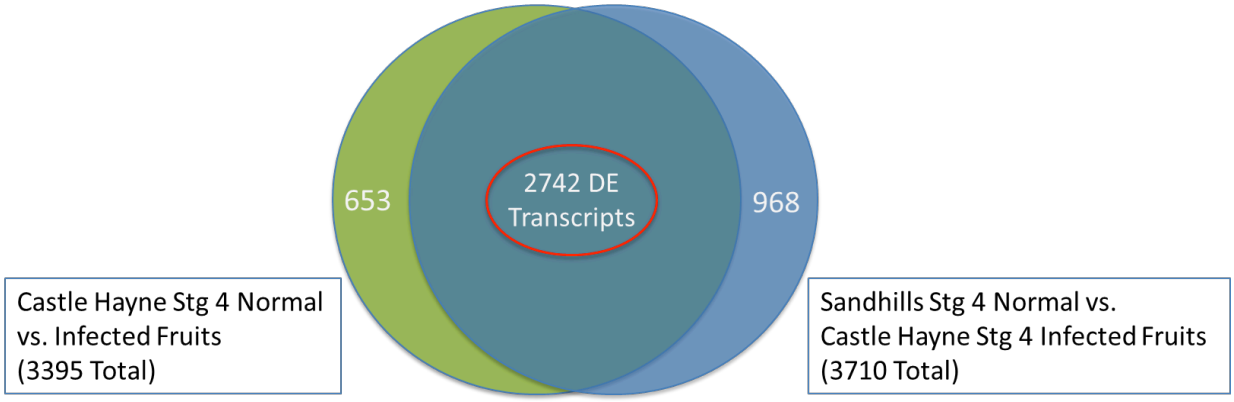


Figure 2. A total of 2742 Transcripts identified in the overlap between the 2 tissue type and across two locations.

Table 2. List of top 10 Gene Ontology terms (gene clusters) that are affected by mummy

GO Term	# Seqs	% Up-Regulated Genes	% Down-Regulated Genes
Defense response	151	75%	25%
Response to jasmonic acid	81	81%	19%
Defense response to fungus	57	77%	23%
Response to salicylic acid	53	81%	19%
Response to chitin	35	86%	14%
Innate immune response	29	83%	17%
Salicylic acid metabolic process	27	85%	15%
Salicylic acid glucosyltransferase (glucoside-forming) activity	25	88%	12%
Chitin binding	24	88%	13%
Jasmonic acid mediated signaling pathway	23	78%	22%

berry

Conclusions

This is the first report of transcriptome analysis of blueberry plants in response to mummy berry. At the last staged of the disease progression when the fungus fully overcome the fruit, the elevated expression of the defense response genes in blueberry in a susceptible cultivar (Arlen) indicates that the pathogen triggers the expression of majority of genes in SAR or ISR pathways; however, Arlen lacks certain resistance genes in response to elicitors and effectors of the pathogen. A relatively few number (30%) of SAR or ISR related genes in blueberry were down regulated in mummified fruit compared to healthy fruit, thus it is possible that the pathogen silenced those key genes SAR and ISR pathways to overcome the innate plant defense system. At the same time

we are investigating the progression of the disease during early stages of flower and flower development to understand how the pathogen is mimicking the pollen grain behavior. It is too early to draw any conclusion about the exact mechanism by which MVC changes its behavior from being a necrotrophic pathogen (during shoot infection) to symbiotic organism (during flower infection) and yet again as a mummifying agent inside the ovaries of the fruit. For the next phase of the project, we will simultaneously inoculate flowers of Arlen and another cultivar that we speculate has inherent resistance to MVC. Subsequently, we will collect RNA at different time points from stylar tissue to identify genes that are differentially expresses during the early stages of infection.

Economic Impact:

Crop loss due to mummy berry has been reported since the 1960's. In 1969, growers in British Colombia, Canada lost 8.1% of their crop due to mummy berry estimating $750,000 [7]. New Hampshire's blueberry producers lost 70-80% of their crop in 1974 [8]. In 1981, mummy berry became an epidemic in Nova Scotia blueberry growing regions destroying 30% of 250 hectares of blueberry fields. In 2002 in no-spray rabbiteye blueberry fields in North Carolina, 70-80% of crop loss was reported [9]. The use of resistant cultivars will save blueberry growers thousands of dollars per year by reducing the cost of fungicide spray. We are the beginning of our path to use marker assisted selection to develop new cultivars that are resistant to mummy berry. This project was a proper start to continue our path toward that goal.

Posters Publications

1. Yow, A., K. Burchhardt, M. Cubeta, **H. Ashrafi**. Identification of Candidate Genes for Mummy Berry Disease Resistance in Blueberry. NCSU Graduate Student Research Symposium. March 22, 2017, Raleigh, NC. Poster presentation (Poster 205).
2. Yow, A., K. Burchhardt, M. Cubeta, **H. Ashrafi**. Identification of Candidate Genes for Mummy Berry Disease Resistance in Blueberry. International Conference on the Status of Plant and Animal Genome Research (PAG XXV). January 14-18, 2017, San Diego, CA. Poster presentation (Poster P0244).
3. Yow, A., K. Burchhardt, M. Cubeta, **H. Ashrafi**. Identification of Differentially Expressed Genes for Mummy Berry (*Monilinia vaccinii-corymbosi*) Resistance in Blueberry. Conference for the American Society for Horticultural Science (ASHS). August 7-11, 2016, Atlanta, GA. Poster presentation (Poster 120).
4. Yow, A., W. Guo, **H. Ashrafi**. Full-Length Transcriptome Sequencing Using PacBio Sequencing in Blueberry. NCSU CALS Stewards of the Future Conference. November 2, 2015, Raleigh, NC. Poster presentation.

Literature Cited

1. Burchhardt KM, Cubeta MA: **Population Structure of the Blueberry Pathogen Monilinia vaccinii-corymbosi in the United States**. *Phytopathology* 2014, **105**(4):533-541.
2. Holst-Jensen A, Kohn L, Jakobsen K, Schumacher T: **Molecular phylogeny and evolution of Monilinia (Sclerotiniaceae) based on coding and noncoding rDNA sequences**. *Am J Bot* 1997, **84**(5):686.
3. Gosch C: **Mummy berry disease (*Monlinia vaccinii-corymbosi*) on highbush blueberries in Europe**. In*: 2006*: International Society for Horticultural Science (ISHS), Leuven, Belgium; 2006: 469-472.
4. Ballington J: **Blueberry Cultivars Released from North Carolina State University Blueberry Breeding Program**. In.: North Carolina State University; 2015.
5. Retamales JB, Hancock JF: **Blueberries**. In: *Crop production science in horticulture series ; 21*. Wallingford, Oxfordshire ; Cambridge, MA : CABI, c2012.; 2012.
6. Cline WO: **An Overview of Blueberry Releases from NCSU - and Other Cultivars of Interest for NC Growers**. In.
7. Pepin HS, Toms HNW: **Economic loss from mummy berry of highbush blue-berry in coastal British Columbia**. *Canada Plant Disease Survey* 1969, **49**(4):105-107.
8. Wallace DB, MacHardy WE, Meader EM: **Control of mummy berry of highbush blueberry in New Hampshire**. *Plant Dis Rep* 1976., **60**:96-101.
9. **Mummy Berry** [http://www.apsnet.org/edcenter/intropp/lessons/fungi/ascomycetes/Pages/MummyBerry.aspx]