ANALYSIS OF CYTOSINE METHYLATION IN SOYBEAN PATHOGEN PHYTOPHTHORA SOJAE

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A Thesis

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*Phytophthora sojae* is plant pathogenic oomycete that targets soybean plants and undergoes a diverse set of life stages specific to its environmental surroundings. Corresponding changes in gene expression occur, although the mechanisms by which genes are differentially expressed and silenced are not fully understood. Methods in regulation of gene expression in *P. sojae* may include various mechanisms at the transcriptional or post-transcriptional level, including DNA CpG methylation. Among the group of regulated genes in *P. sojae* are avirulence genes, which encode effector proteins that are utilized during infection. Hundreds of *Avr* genes are present in the *P. sojae* genome, although their expression is not uniform across different races. In this study, we investigate the effects of low levels of the cytosine methyltransferase inhibitor 5-aza-2’-deoxycytidine on growth and pathogenicity, in conjunction with qRT-PCR analysis of the housekeeping gene, actin, and the avirulence gene, *Avr1a*. 
Dedicated to my mother, father, stepmom, brother, sister, and my dear friend, Maya.
ACKNOWLEDGEMENTS.

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INTRODUCTION

*Phytophthora sojae*

*Phytophthora sojae* is a soil-borne pathogen of soybean plants, a eukaryote belonging to the diverse class, oomycota. Oomycete pathogens differ greatly even among themselves, individual species ranging from saprophytic to pathogenic and spanning a wide range of hosts extending from plants, to fish, to mammals. Oomycetes were long classified as fungi due to morphology-based groupings, although phylogenetic testing now categorizes them as closer-related to diatoms than fungi (Tyler, 2006). In addition to phylogenetic classification, oomycetes have several important physiological attributes that distinguish them from fungi.

The seemingly common hyphal structures that initially relegated oomycetes to the nomenclature of ‘lower fungi’ actually differ from their fungal counterparts in that oomycete hyphae are aseptate and linked through gap junctions, whereas the hyphae of true fungi can be of a single cell or septate, with one or more nuclei per compartment (Judelson & Blanco, 2005). Because oomycete cell wall composition includes cellulose and beta glucans instead of comparatively large amounts of chitin as fungi do, fungicides targeting chitin synthesis pathways have little to no effect in controlling or preventing infection by oomycetes in a field application. Another differentiating feature from fungi includes the beta glucan mycolaminarin, an energy storage carbohydrate that that is also found in kelps and diatoms (Erwin, *et al.*, 1983). While motile spores are rare in fungi, zoospores are nearly universal in oomycetes. These zoospores range in shape and dispersal, are largely chemotaxic, and utilize both a tinsel (anterior) and a whiplash (posterior) flagella (Judelson & Blanco, 2005; Walker & van West, 2007).

*Phytophthora sojae* is an oomycete pathogen with a narrow host range, targeting mainly
soybean plants. The United States is a major exporter of the cash crop, producing $37.6 billion worth of soybeans in 2011 (USDA, 2012). *P. sojae* is estimated to result in the loss of approximately 43 million bushels of soybeans per year in the United States (Koenning, 2010), and $200 million of economic loss per year in the United States’ northern Midwest alone (Tyler, 2007).

**Plant - Pathogen Interactions**

A wide variety of mechanisms are utilized by pathogens to successfully infect and evade detection by host plants, determined by pathogen strategy and host specificity. A pathogen’s access to the nutrients in a plant is achieved by several methods, including pre-existing tissue injury, utilization of stomatal openings, cellular penetration, and root invasion (Faulkner & Robatzek, 2012). In all these methods, the pathogen may be identified or disabled through specialized receptors located in the plasma membrane or the cytosol of the host plant.

Pattern Recognition Receptors (PRRs) located in the plasma membrane of host plants are largely characterized by Leucine-Rich Repeats (LRRs) or Lysine Motifs (LysM). These receptors act as the plant’s indication of a pathogenic presence, signalling for downstream events when targeted Pathogen-Associated Molecular Patterns (PAMPs) are detected. This reaction can be silenced by effector proteins encoded by avirulence (*Avr*) genes, allowing the pathogen to remain undetected during infection by preventing activity of PRRs or interrupting their signals (Ellis, *et al*, 2006). This type of interference in host cell immunity is referred to as PAMP-Triggered Immunity (PTI).

Resistance genes (R-genes) encode cytosolic receptors that recognize effectors or other signs of pathogen presence when PRRs have been circumvented. These receptors are composed of Nucleotide Binding (NB) and LRR domains, and co-evolve closely with their corresponding R-genes. This results
in a highly specialized gene-for-gene system in which a pathogenic strain can be specific to a very narrow host range, even a single plant cultivar (Faulkner & Robatzek, 2012).

**Infection by *Phytophthora sojae***

Soybeans are susceptible to infection by *Phytophthora sojae* throughout their lifespan on any part of the plant, but primarily at the root (Tyler, 2006). *P. sojae* spreads by growth of mycelium and release of zoospores, primarily in wet conditions such as flooded fields after heavy rain, especially those with poor drainage. Successful infection results in seed decay, damping off in seedlings, and root or stem rot in mature plants.

Encystment on soybean tissue occurs when a sporangium or zoospore comes in contact with the plant, and serves as the entry location for the pathogen. Sporangia may infect root tissue directly, but it is much more common for infection to occur as a result of zoospore release (Tyler, 2007). Once the cyst has been formed on the surface of the plant tissue, a germ tube forms and the attached appressorium penetrates the cell wall utilizing turgor differences. Once inside the cell wall, haustoria will grow between cells, collecting nutrients and releasing effector proteins while surrounded by a host-derived extra-haustorial membrane (Whisson, 2007).

Over 400 effectors predicted in *Phytophthora sojae* contain an Arg-X-Leu-Arg (RXLR) motif, a feature common to many plant pathogenic oomycetes (Shan, *et al.*, 2004). Effectors containing this motif contain three conserved regions: an N-terminal secretion signal, the RXLR conserved sequence, and a C-terminal effector domain (Oliva, 2010). Some RXLR proteins also contain a second motif, Asp-Glu-Glu-Arg (dEER) following the RXLR region, as well as W and Y motifs in the C-terminal effector domain (Rehmany, 2005; Dou, *et al.*, 2008). The RXLR motif is necessary for the
transit of effector proteins into host cells after their release from haustoria (Whisson, 2007).

**Phytophthora sojae Avirulence Genes**

*Avr1a* and *Avr1b* are well-categorized avirulence genes in the *Phytophthora sojae* genome that exhibit RXLR and dEER motifs. Several races of *Phytophthora sojae* contain copies of the *Avr1a* and *Avr1c* genes but silence them by an undetermined mechanism (Na, *et al.*, 2014). There are three copies of *Avr1a* in *P. sojae*, two being identical (*Avh275a, Avh275b*), and the third (*Avh72*) being a pseudogene with a premature stop codon contained within the sequence (Qutob, 2009). The *Avr1c* gene is a close paralog of *Avr1a*, sharing a near-identical N-terminal region resulting classification as an additional copy of *Avr1a* at various times (Na, *et al.*, 2014). *Avr1b* is located at two locations in the genome, *Avr1b*-1 and *Avr1b*-2, and is associated with soybean resistance gene, *Rps1b*. The first copy of the gene encodes the effector protein, and the second plays a role in the accumulation of *Avr1b*-1 mRNA (Shan, *et al.*, 2003).

*P. sojae* expressing either *Avr1c* or *Avr1a* will trigger programmed cell death (PCD) in soybean plants containing *Avr1c*’s corresponding R-gene, *Rps1c*. This dual specificity is not observed in plants with *Rps1a*; only expression of *Avr1a* will induce PCD (Na, *et al.*, 2014). PCD can be suppressed by *P. sojae* expressing *Avr1b* due to C-terminal W and Y motifs that interact with targeted host proteins (Dou, *et al.*, 2008). Evidence of outcrossing in *P. sojae* races resulting in virulence against soybean plants of more *Rps* genes has been recorded. An instance of this includes a novel *P. sojae* race isolated in a location where race 4 (virulent on *Rpsla, lc and 7*) and race 15 (virulent on *Rps3a and 7*) had been previously documented that was virulent against soybean plants containing the combined *Rps* genes of both race 4 and race 15 (Whisson, *et al.*, 1994).
DNA Methylation

DNA cytosine methylation is established as a regulatory epigenetic mechanism present in many eukaryotic organisms including vertebrates, plants, and fungi (Finnegan, et al, 1998). Methylation on CpG islands in the promoter region of a gene can result in gene silencing in many organisms. Regulatory cytosine methylation is very common and widely present in mammal and plant cells, occurring in CpG islands in mammals, as well as CpG, CpHpG, and CpHpH locations in plants.

Through the normal methylation and demethylation of various genes, an organism may go through various physiological changes related to development, environmental change, pathogenesis, etc. Without the hereditary inheritance of methyl patterns, cells may be left hemimethylated, resulting in the abnormal expression of several genes otherwise regulated by CpG methylation. Abnormal hypermethylation is associated with many types of cancer, partially the result of regulatory genes being suppressed. Although studies have been conducted on the regulation of individual genes, it is presently unknown whether the soybean pathogen *Phytophthora sojae* utilizes CpG methylation in regulation of its genome as a whole (van West, et al, 2008).

5-Aza-2’-Deoxycytidine

The synthesis of 5-azacytidine and 5-aza-2’-deoxycytidine was first described in 1964, originally the indirect byproduct of studies concerning *E. coli* cells grown in sub-bacteriostatic concentrations of 5-azauracil in which unexpected derivatives were observed (Piskala & Sorm 1964). Almost twenty years later, the effect of these two chemicals on certain methyltransferase activity and therefore cytosine C5 methylation was observed (Lee, et al, 1976; Lu, et al, 1980; Taylor, et al, 1982). 5-aza-2’-deoxycytidine is at least 10-fold more toxic to cultured cells than 5-azacytidine, so it is
necessary to ascertain appropriate concentrations for effective treatment of DNA (Flatau, et al, 1984; Momparler, et al, 1984). When implemented at low enough concentrations, 5-aza-2’-deoxycytidine can induce DNA hemimethylation at DNA cytosine C5 locations, and therefore, increased gene expression in organisms that utilize methylation as a regulatory mechanism.

5-aza-2’-deoxycytidine acts as a cytosine analogue, being incorporated into DNA strands during synthesis (Vesely, et al, 1978). Due to the presence of a nitrogen instead of a carbon at position 5 on the pyrimidine ring of the molecule, the enzyme methyltransferase is unable to dissociate after binding, and can therefore no longer methylate CpG locations in the genome (Christman, et al, 1985). 5-aza-2’-deoxycytidine has since been approved and implemented as a therapeutic agent under the name decitabine to combat the hypermethylation of regulatory genes characteristic of various types of cancers (Christman J., 2002).

With this type of treatment, genes previously silenced via cytosine C5 methylation can be expressed again as new copies of the DNA strands will be less likely to be methylated. For cancer treatments this means the renewed expression of important regulatory proteins necessary for proper cell function, but the same concept can be applied to a system in which cytosine methylation is not a known silencing mechanism. When treated with an appropriate concentration of 5-aza-2’-deoxycytidine, gene expression and possibly observable phenotypes should be altered compared to a mock sample if DNA cytosine C5 methylation is a regulatory strategy employed by the organism in question (Tamame, et al, 1983; Barnes, et al, 2014).
PURPOSE

We hypothesize that CpG methylation plays a role in *Phytophthora sojae* gene regulation. The purpose of the experiment is to ascertain the presence and effect of cytosine methylation on gene regulation in *Phytophthora sojae*. This will be tested 1) by pathogenicity assay of mycelia grown in the presence or absence of 5-aza-2’-deoxycytidine on soybean hypocotyls; 2) through the use of qRT-PCR on cDNA produced using different concentrations of a DNA methylation inhibiting agent, 5-aza-2’-deoxycytidine.
METHODS AND MATERIALS

*Phytophthora sojae* Growth Conditions and Measurements

*Phytophthora sojae* was grown in dark conditions at room temperature on V8 agar (200ml V8 vegetable juice, 2.5g CaCO₃, and 15g bacto-style agar per liter of ddH₂O) with 5-aza-2’-deoxycytidine at final concentrations of 0.0µM, 3.1µM, 6.25µM, and 12.5µM. For plates designated for growth measurement, treatments were produced with five replicates. Growth measurements were made once daily for four days to ascertain appropriate concentrations for 5-aza-2’-deoxycytidine treatment for *P. sojae*. In doing so, significant differences in gene expression could be more reliably attributed to methyltransferase inhibition instead of direct toxicity to cells.

For RNA extractions, *P. sojae* was grown in the dark on V8 agar with 0.0µM, 3.1µM, and 6.25µM concentrations of 5-aza-2’-deoxycytidine. Before plugs were transferred to plates being used for RNA extraction, a membrane filter was placed over the agar, allowing mycelia to access nutrients from the agar and providing a quick method to harvest mycelia. After seven days’ growth, the mycelia were removed from the membrane of each plate and immediately frozen in liquid nitrogen. Samples from the three treatments were then crushed and stored at -80° until used for RNA extraction.

Zoospore Induction

Mycelia used for zoosporogenesis was grown in dark conditions at room temperature on clarified V8 agar (200ml V8 vegetable juice and 15g bacto-style agar per liter of ddH₂O) at 5-aza-2’-deoxycytidine concentrations of 0.0µM, 3.1µM, 6.25µM, and 12.5µM. After four days of growth, plates were flooded with pH7 ddH₂O overnight in dark conditions to induce sporangia.
development. Following this incubation, plates were rinsed with fresh water every 20 minutes. Upon release, zoospores were pipetted onto a depression slide for observation.

RNA Extraction

Mycelia used for RNA extraction was taken from three V8 agar plates per concentration of 5-aza-2’-deoxycytidine, frozen in liquid nitrogen, and crushed. Total RNA was isolated from 100 mg frozen and crushed *P. sojae* mycelia using the RNeasy Plant Mini Kit (QIAGEN, CA) according to manufacturer’s instructions. Two elutions were made, each with 30µl RNase free water. RNA concentration and purity were determined using NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, MA) and visualized by gel electrophoresis to ensure degradation had not occurred.

Infected soybean hypocotyls were selected from mock as well as treatment groups for RNA extraction from infected tissue. Infected regions were isolated and immediately frozen in liquid nitrogen, and the above protocol for RNeasy Plant Mini Kit (QIAGEN, CA) was followed.

First-Strand cDNA Synthesis

cDNA synthesis was conducted using SuperScript III Reverse Transcriptase (Invitrogen, CA). 1µl of oligo(dT)\textsubscript{20} primer, 250 ng total RNA, 1µl of 10 mM dNTP mix (10mM of dCTP, dGTP, dATP, and dTTP each), and 8.5µl of RNase-free H\textsubscript{2}O were mixed on ice and incubated at 65°C for 5 minutes and then immediately put on ice for two minutes to prevent secondary structure formation. Following this incubation 4µl of 5x First-Strand Buffer, 1µl of 0.1M DTT, 1µl of 0.01% DEPC-treated water and 1µl of SuperScript III RT were added to the mixes over ice before being heated to 25°C for 5 minutes, 50°C for 45 minutes, and finally 70°C to inactivate the reaction. Samples were then stored at -20°C
until utilized for qRT-PCR.

**Gene Candidates**

Forward and reverse primers were designed for gene amplification through qRT-PCR. An avirulence gene (*Avr1a*) was chosen as a candidate for amplification and the housekeeping gene *Actin* was included for normalization.

**Table 1. Gene Candidates for qRT-PCR:** Forward and reverse primers were designed to quantify the mRNA copies present of selected genes in samples of RNA.

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<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>Actin</td>
<td>AGCGTATGACCAAGGAGCTG</td>
<td>TTCGAGATCCACATCTGCTG</td>
</tr>
<tr>
<td>Avr1a</td>
<td>CGATGTCCTCACCACTGAGA</td>
<td>GCTCGTTACCCTTCATTGT</td>
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**Quantitative Reverse Transcription PCR**

qRT-PCR was conducted in 15µl reactions containing the following: 7.5µl of Fast SYBR Green Master Mix (Applied Biosystems, CA), 1µM gene specific primer, 250 ng of template DNA, and 4.7µl of ddH₂O. Each gene was amplified in triplicate from each of the three 5-aza-2’-deoxycytidine cDNA samples, as well as a No Template Control (NTC) tube for each set of cDNA, resulting in 16 individual tubes per amplified gene.

**Pathogenicity Assay**

Williams Soybean seeds were sterilized with subsequent washes of 2% Clorox, 70% EtOH, and distilled water. The seeds were then placed on moist autoclaved paper towels, covered, and stored
in the dark for four days to germinate. Hyphal plugs of *Phytophthora sojae* hyphae grown in the presence of 5-aza-2’-deoxycytidine at concentrations of 0.0µM, 3.1µM, and 6.25µM were placed onto the sterile soybean hypocotyls and left for infection.

State of infection was observed after 16 and 24 hours, at which time Lactophenol Cotton Blue stain (Carolina, NC) was used to visualize *P. sojae* mycelia to differentiate from plant material. Whole mounts and cross sections of infected hypocotyls were observed under microscope.
RESULTS

Effect of 5-aza-2’-deoxycytidine Treatment on Mycelia Growth

Measurements of Phytophthora sojae growth were conducted in five replicates per treatment of 5-aza-2’-deoxycytidine. The diameter of mycelial growth was measured over a period of four days to ensure concentrations of 5-aza-2’-deoxycytidine in the growth media was not directly toxic to cells. The measurements for the individual plates were averaged for each day and plotted below (Figure 1). There was no significant difference in the growth rate of Phytophthora sojae between treatments, indicating that the concentrations of 5-aza-2’-deoxycytidine incorporated into the media do not have a direct toxicity that would inhibit growth. 5-aza-2’-deoxycytidine concentrations of 0.0µM, 3.1µM, and 6.25µM were selected for later work based on these results.

Figure 1. Mycelia growth rates in the presence of 5-aza-2’-deoxycytidine at concentrations of 0.0µM, 3.1µM, and 6.25µM: No significant difference in growth was observed between control and treatment groups. ANOVA results produced a p-value of 0.92.
Zoospore Swim Pattern

*Phytophthora sojae* grown on Clarified V8 agar plates containing 5-aza-2’-deoxycytidine at concentrations of 0.0µM, 3.1µM, and 6.25µM were flooded overnight after four days’ growth, then rinsed every 20 minutes with fresh pH7 ddH$_2$O for eight hours. Resulting zoospores were pipetted onto depression slides and videos were recorded at 40x total magnification. Two types of swim patterns were observed; pivoting, typical of normally functioning zoospores, and circuitous. Although zoospores from both control and plates treated with 5-aza-2’-deoxycytidine displayed normal and circuitous swimming patterns, those from the treated plates displayed the circuitous swimming pattern more often.

![figure2.png](https://example.com/figure2.png)

**Figure 2. Zoospore Swim Patterns:** Zoospores displayed variation in swim patterns at increased concentrations of 5-aza-2’-deoxycytidine. Zoospores produced on plates without 5-aza-2’-deoxycytidine were more likely to display swim patterns typical of *P. sojae*, while those grown at 12.5µM more often displayed a swim tightly-wound, circular swim pattern.
Pathogenicity Assay of Soybean Hypocotyls

Soybean seeds were allowed to germinate in dark, moist conditions at room temperature for four days before hypocotyls were exposed to hyphal plugs of *Phytophthora sojae* grown at the 0.0µM, 3.1µM, and 6.25µM of 5-aza-2’-deoxycytidine. Hypocotyls were then returned to above conditions for an additional sixteen hours while infection occurred and then observed as whole mounts and cross sections by light microscope. *Phytophthora sojae* was dyed with Lactophenol Cotton Blue (Carolina, NC) to differentiate hyphae from plant tissue.
Figure 3. Infection by *Phytophthora sojae* on soybean hypocotyls: Soybean hypocotyls were inoculated with *P. sojae* hyphae grown on media containing 0.0µM, 3.1µM, and 6.25µM of 5-aza-2’-deoxycytidine. At 16 hours post-infection, *P. sojae* hyphae were dyed with Lactophenol
Cotton Blue (Carolina, NC), allowing for visual differentiation from plant tissue. Row 1 shows cross section and whole mounts of hypocotyl infected with control hyphae, Row 2 with hyphae grown in the presence of 3.1µM 5-aza-2’-deoxycytidine, and Row 3 with hyphae grown in the presence of 6.25µM 5-aza-2’-deoxycytidine. Each scale bar is representative of one micrometer.

RNA Extraction

Total RNA was extracted from both mycelia grown on V8 agar and soybean hypocotyl tissue infected with hyphae grown in the presence of 5-aza-2’-deoxycytidine concentrations of 0.0µM, 3.1µM, and 6.25µM. Total RNA was then measured for concentration and purity by spectrophotometry (Tables 2 and 3). To ensure degradation had not occurred, first and second elutions were run on a 1.5% agarose gel (Figures 4 and 5).

Table 2. Purity and Concentration of Total RNA Extracted from Mycelia Grown on V8 Media: Prior to cDNA synthesis, purity and concentration of first (RNA 1) and second (RNA 2) elutions of total RNA were measured via NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, MA).

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<tr>
<td>3.1µM Myc RNA 1</td>
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</tbody>
</table>
Figure 4. Gel electrophoresis of Total RNA from Mycelia Grown on V8 Media Run on 1.5% Agarose Gel: Approximately 200-450 ng of total RNA produced from mycelia grown in different concentrations of 5-aza-2'-deoxycytidine were run on a 1.5% agarose gel to ensure degradation of transcripts had not occurred. Lanes 1-3 contain 0.0µM Myc RNA 1, 3.1µM Myc RNA 1, and 6.25µM Myc RNA 1, respectively. Lanes 4-6 contain 0.0µM Myc RNA 2, 3.1µM Myc RNA 2, and 6.25µM Myc RNA 2, respectively.
Table 3. Purity and Concentration of Total RNA Extracted from Infected Tissue: Prior to cDNA synthesis, purity and concentration of first (RNA 1) and second (RNA 2) elutions of total RNA from susceptible soybean hypocotyl tissue were measured via NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, MA).

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>260/280</th>
<th>ng/ul</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0µM Infection RNA 1</td>
<td>2.15</td>
<td>605.8</td>
</tr>
<tr>
<td>3.1µM Infection RNA 1</td>
<td>2.14</td>
<td>667.3</td>
</tr>
<tr>
<td>6.25µM Infection RNA 1</td>
<td>2.08</td>
<td>428.4</td>
</tr>
<tr>
<td>0.0µM Infection RNA 2</td>
<td>2.13</td>
<td>55.5</td>
</tr>
<tr>
<td>3.1µM Infection RNA 2</td>
<td>2.11</td>
<td>256.2</td>
</tr>
<tr>
<td>6.25µM Infection RNA 2</td>
<td>2.14</td>
<td>143.4</td>
</tr>
</tbody>
</table>
Figure 5. Gel electrophoresis of Total RNA from Infected Tissue Run on 1.5% Agarose

Gel: Approximately 200-300 ng of total RNA produced from soybean hypocotyl tissue infected with hyphae grown in the presence of different concentrations of 5-aza-2’-deoxycytidine were run on a 1.5% agarose gel to ensure degradation of transcripts had not occurred. Lanes 1-3 contain 0.0µM Infection RNA 1, 3.1µM Infection RNA 1, and 6.25µM Infection RNA 1, respectively. Lanes 4-6 contain 0.0µM Infection RNA 2, 3.1µM Infection RNA 2, and 6.25µM Infection RNA 2, respectively.

Quantitative Reverse Transcription PCR

cDNA produced from both directly from mycelia grown at 0.0µM, 3.1µM, and 6.25µM 5-aza-2’-deoxycytidine and from soybean hypocotyl tissue infected by hyphal plugs grown in the same conditions were used to quantify the transcripts of two candidate genes: Actin, and Avr1a. A significant decrease in expression was observed for gene Avr1a with treatment by 5-aza-2’-deoxycytidine in cDNA from both mycelia and infected tissue.
Figure 6. Relative fold change of *Avr1a* expression in *Phytophthora sojae* mycelia grown in media with concentrations of 5-aza-2'-deoxycytidine by qRT-PCR: cDNA synthesized from mycelia grown in the presence of three concentrations of 5-aza-2'-deoxycytidine was amplified by a set of primers specific to the *Phytophthora sojae* *Avr1a* gene and normalized by the housekeeping gene, actin. Bars indicate ± 1.0 Standard Error of the Mean (SEM).
Figure 7. Relative Fold Change of *P. sojae Avr1a* Expression from Infectious Tissue at Concentrations of 5-Aza-2’-Deoxycytidine, 16 Hours Post Infection, by qRT-PCR: cDNA synthesized from susceptible soybean hypocotyl tissue infected with hyphae grown in the presence of three concentrations of 5-aza-2’-deoxycytidine was amplified by a set of primers specific to the *Phytophthora sojae Avr1a* gene and normalized by the housekeeping gene, actin. Bars indicate ± 1.0 Standard Error of the Mean (SEM).
DISCUSSION

*Phytophthora sojae* is a pathogen that successfully survives and infects its susceptible host through several specialized life stages and the targeted use of various genes. There are numerous strategies that *P. sojae* may utilize to regulate its genome at transcriptional or post-transcriptional levels to achieve the formation of different tissues during development and the expression of appropriate genes reflecting its environmental conditions.

In this study, the cytosine methyltransferase inhibitor 5-aza-2’-deoxycytidine was incorporated into the media upon which *Phytophthora sojae* was grown. 5-aza-2’-deoxycytidine is a common chemotherapeutic agent and is well-studied in cancer research under the name decitabine for its efficacy in re-activating regulatory genes that are commonly silenced in cancerous cells (Christman, 2002). The concentration at which 5-aza-2’-deoxycytidine is applied to cells in cancer and other epigenetic research varies widely due to the necessity of finding appropriate context-specific concentrations that will yield effective treatment without inducing cytotoxicity (Momparler, 2005).

Previous work found that in the presence of high concentrations of 5-aza-2’-deoxycytidine, *Phytophthora sojae* hyphal growth slows and eventually halts after approximately twenty-five subcultures (Benson, 2012). The amount of 5-aza-2’-deoxycytidine used in this study was consecutively lowered by half until concentrations at which cytotoxic effects, approximated by measuring hyphal growth, were avoided while physiological changes such as differences observed in zoospore swim pattern were still observed. By this method, the concentrations of 6.25µM and 3.1µM 5-aza-2’-deoxycytidine were deemed appropriate for epigenetic studies in *Phytophthora sojae*, and selected for the pathogenicity assay and *Avr1a* qRT-PCR study.
Genetic mapping of avirulence genes revealed copy number variation in \textit{Avr1a} homologs among races of \textit{Phytophthora sojae}. Concurrent data from southern blot analysis also showed diversity in transcript accumulation for homolog \textit{Avh275}, although the regulatory mechanism in this context is unknown (Qutob, \textit{et al}, 2009). Similar silencing or weak expression is seen in a wide variety of avirulence genes and may be advantageous in that the loss in expression of a single \textit{Avr} could help evade plant detection by corresponding R-genes, but would not significantly decrease pathogenicity on its own (Wang, \textit{et al}, 2011). During infection of soybean hypocotyls with \textit{P. sojae} grown in the presence of low concentrations of 5-aza-2’-deoxycytidine, increased pathogenicity was consistently observed in treated samples when compared to mock samples. This suggests an increase in effector expression induced by demethylation of an unknown number of corresponding avirulence genes.

Past research has suggested that gene regulation by cytosine methylation is not utilized in the \textit{Phytophthora} genus. A study conducted in \textit{Phytophthora infestans} to determine the mechanism by which the elicitin gene \textit{inf1} remained silenced after a prolonged period of time included methylation-specific restriction assays and bisulfite sequencing of the gene, but concluded that silencing was instead the result of chromatin remodeling (van West, \textit{et al}., 2008). Although determined to be unrelated to \textit{inf1} regulation, cytosine methylation may play a role in regulating other genes within the \textit{Phytophthora} genus. A later study found homologs in \textit{P. infestans} related to gene silencing mechanisms including RNAi, histone modification, and chromodomain proteins, suggesting other avenues of gene regulation in \textit{Phytophthora} (Vetukuri, \textit{et al}., 2011). In this paper, the author’s parameters for finding homologs included genes related to a wide range of regulatory mechanisms including histone acetylation and methylation, but not cytosine methylation. In addition, bioinformatic
prediction by itself may prove to be inadequate in determining the presence of potential methylases in *Phytophthora* genomes if sufficient homology for detection is not present.

These studies, while providing valuable insight into the management of *Phytophthora infestans* genome, do not provide definitive evidence that *Phytophthora sojae* does not regulate its genome at least partially through DNA cytosine methylation. qRT-PCR analysis of *Avr1a* showed a decrease in expression when mycelia (from both V8 and infection) were grown in the presence of both the 3.1µM and 6.25µM concentrations of 5-aza-2’-deoxycytidine when compared to non-treated samples. A decrease in methyl groups would normally lead to an increase of gene expression, but the opposite may also be obtained through a more indirect route. The decrease observed in *Avr1a* expression may be due to indirect regulation via DNA demethylation (and therefore induced expression) of a separate suppressor that acts directly upon the *Avr1a* gene, suggesting an interaction of regulatory mechanisms. Future research involving bisulfite sequencing or RNAseq data would provide a more complete picture of the role DNA cytosine methylation plays in *Phytophthora sojae* gene regulation.
REFERENCES


Spangler, M. 2012. *Cytosine methylation of Phytophthora sojae by methylated DNA immunoprecipitation*. 


