Exploration of Epigenetic Modifications in Taxus Cell Culture using Global Demethylating Agents

A Major Qualifying Project (MQP)

Submitted to the Faculty of

WORCESTER POLYTECHNIC INSTITUTE

In partial fulfillment of the requirements for the

degree of Bachelor of Science

in Chemical Engineering

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Date: 6 May 2021

Report Submitted to:

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I. Abstract

Paclitaxel is a widely used chemotherapeutic drug derived from yew trees *(Taxus* species). Meeting world demand for paclitaxel has historically been difficult, as it is produced in very low concentrations within the bark of the yew tree. Plant cell culture is one sustainable method for paclitaxel production, but as cell lines get older and are continuously subcultured, production decreases. This decreased production is correlated with increased DNA methylation, which is an epigenetic mechanism that downregulates gene expression. This MQP sought to find a global demethylating agent that could reverse this to maintain high paclitaxel yields, even in cultures that have been subcultured for many years. Two cell lines were treated with either 5-azacytidine or zebularine (two global demethylating agents with different mechanisms of action) at either 100μM or 200μM. Half of the flasks were elicited with methyl jasmonate, and half were mock-elicited. Levels of paclitaxel and its precursors, as well as flavonoids and phenolics, were sampled and measured weekly over the course of the 21-day long experiment. Treatment, while visually looking to positively correlate with paclitaxel production, was found to have no statistically significant effect on paclitaxel production. There was also no effect on the production of paclitaxel precursors 10-deacetylbaccatin and baccatin. It was further found that there was no difference between 5-azacytidine and zebularine or between the different concentrations. Notably, there was also no difference between the elicited and mock-elicited flasks. In addition, treatment had no discernable effect on flavonoid and phenolic production.

II. Acknowledgements

Our team would like to thank the following individuals that helped make this project possible. First, we would like to thank our project advisor, Professor Susan C. Roberts, for her insight and guidance with the project. Second, we would like to thank Cassandra M. Brzycki. She taught and assisted us with all lab procedures that we needed to complete this project. We are extremely thankful for her willingness to help throughout every step of the project and her endless patience as we learned these research techniques.

III. Table of Contents

IV. Table of Figures

V. Table of Tables

VI. Introduction

Secondary Metabolites

Plants produce many different compounds to protect themselves against environmental stressors, predators, and pathogens [1]. Many compounds of interest are classified as secondary metabolites, which are compounds not directly involved in the growth or development of an organism. These specialized organic compounds are highly diverse and have played an essential role in medicinal drug discoveries, new food additives, cosmetic dyes, and fragrances. For instance, one of the more widely used chemotherapeutic drugs, paclitaxel, comes from plants. Artemisinin, an anti-malarial drug, is also a plant natural product. However, there are several obstacles to their commercial-scale production.

Secondary metabolites often exist in low concentrations at their natural source, and many plants have already been over-harvested to meet demand. This often leads to species endangerment, which is unsustainable and harmful to the environment. Additionally, plant species can have prolonged growth periods with fluctuations in production of their secondary metabolites, making regrowth of the natural source impractical as well [2]. As for the complete chemical synthesis approach, while appealing for simple compounds, this approach is not reasonable for many more complex secondary metabolites. These metabolites can have complex biochemical pathways that either require expensive precursors, can only be produced using synthesis schemes with very low yields, or cannot be recreated altogether. This makes the process economically infeasible for large scale production [3]. Another method is called heterologous production. This involves taking part of the DNA from one organism and inserting it into the DNA of another. This allows for the second organism to produce the natural products of the first one and can be engineered to be done at higher concentrations. Plant cell culture,

which involves taking material from the plant and transferring the cells to laboratory cultures, is yet another method used, but suffers from low concentrations.

Currently, the most effective strategy for production for many complex secondary metabolites is the use of plant cell culture, which is more sustainable and economical. In plant cell cultures, harvested cells are transferred to and propagated in a specific media. The original flasks containing the cells can then be subcultured indefinitely, allowing for a sustainable production source. Unlike natural harvest, plant cell cultures produce consistent yields and can be grown in suspension cultures similar to yeast or bacterial cells, making large scale bioprocessing operations easier to control. With such flexibility in controlling the growth environment, it is also easier to address the next obstacle in large-scale production: increasing the secondary metabolite yield [1].

Metabolic Engineering and Elicitation

The most common method for increasing secondary metabolite yield in biological systems is metabolic engineering, or the targeted optimization of a cell's regulatory and genetic processes. With metabolic engineering, specific biochemical reactions can be directly modified using genome-editing techniques to improve production of specific compounds of interest [4]. This strategy has historically had great success in improving industrial-scale plant cell processes, improving economic feasibility and accessibility of these compounds. There are three primary tools commonly used in plant metabolic engineering in accomplish this: pathway overexpression, transcription factor modification, and elicitation [5].

Pathway overexpression is a commonly used and effective metabolic engineering technique, which increases the synthesis of desired metabolites by over-expressing the rate-influencing steps of the biosynthetic pathway. In plant cell culture, a strong promoter is typically

used to transform the gene of interest within a vector. This has been shown to nearly double the production of certain secondary metabolites, such as with the overexpression of the DBAT gene in *Taxus* cell culture [5].

Another metabolic engineering technique, transcription factor modification, is where groups of transcription factors are intentionally overexpressed or under expressed to control the rate of gene transcription. Transcription factors operate by binding to the DNA sequence, to either repress or activate specific cascades of gene expression. Thus, intentionally modifying these transcription factors associated with secondary metabolite gene-expression has been shown to increase the production of these secondary metabolites. This can occur by either under expressing a gene that represses secondary metabolite production or upregulating a gene that is already involved in activating secondary metabolite synthesis [5,6].

Elicitation is another metabolic engineering method, where abiotic or biotic agents are added to the plant cell growth medium to induce stress responses in the cells. This induced stress allows for manipulation of metabolic pathways, aimed to characterize the cell's response to environmental factors and trigger increased production of specific stress-associated secondary metabolites. Depending on the type of elicitor, the biosynthetic pathways linked to production can be activated by simulating a defense response in the cells. These elicitors trigger signal transduction cascades, as shown in Figure 1 below, which activate the synthesis of transcription factors that can regulate gene expression [7]. Quick access to this regulation is particularly attractive for commercial applications, as production can be greatly increased without the use of numerous or expensive precursors or genetic engineering [8].

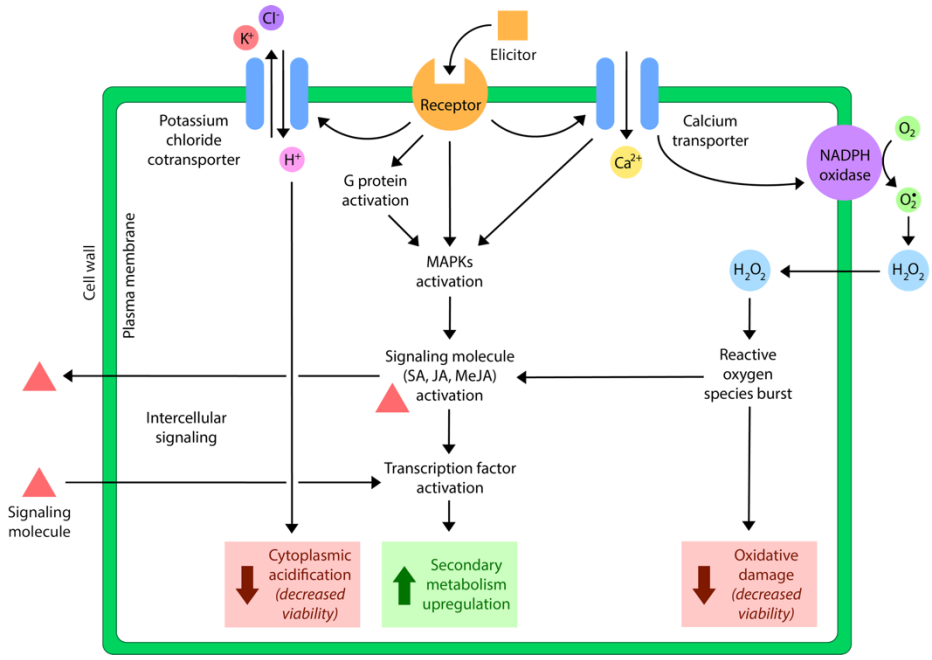

Figure 1: *Diagram of plant cell elicitation signal transduction pathway (sourced from [5])*

The effectiveness of elicitation differs depending on a variety of factors, including the class and type of elicitor, elicitor concentration, duration of elicitor exposure, media nutrient composition, and culture age. Plant cells can be hypersensitive to these variables, and tests to determine the highest possible yield without inducing adverse effects such as cell death or decreased growth rates are particularly important [9]. Overall, despite its potentially negative effects, elicitation is currently one of the most widely used methods for metabolic engineering due to its practicality at a large-scale.

Epigenetics

Despite the proven benefits of these metabolic engineering techniques in increasing secondary metabolite yield, to achieve long-term success at industry levels plant cell culture epigenetics must also be considered. Over time, epigenetic mechanisms, which play an essential role in regulating gene expression, can decrease yields of secondary metabolites in plant cells as they adapt to suspension culture. Thus, to maintain high production it is necessary to both

understand and ultimately control epigenetic mechanisms [5]. The research of and interest in epigenetics in plant cell culture has grown significantly over the course of the last decade to try and accomplish this.

The term “epigenetics,” originally coined by Conrad Waddington in the early 1940s, has been defined in multiple and often inconsistent ways. At its conception, Waddington centered his definition around the decoupling of the genotype and phenotype and the realization that some sort of regulatory mechanism must exist between the two [10]. As more research was done on the topic, this would come to be redefined, notably by Robin Holliday in the 1990s. In a paper entitled “Epigenetics: An Overview”, Holliday offered two definitions of epigenetics; one, “the study of the changes in gene expression, which occur in organisms with differentiated cells, and the mitotic inheritance of given patterns of gene expression”, and “nuclear inheritance, which is not based on differences in DNA sequence” [11]. While this definition was similar to Waddington’s, it notably added the concept of inheritance. Epigenetics shortly after became a field for phenomena that could not cleanly fit into other genetic explanations. The definition was streamlined by Wu and Morris (2001) into a more modern definition to say that epigenetics is “the study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail change in DNA sequence” [12]. Some common mechanisms of epigenetic modification that are of interest include DNA methylation, histone modification, and RNA interference, as they have proven roles in regulating gene expression [13]. The most widely accepted of these is DNA methylation, and its regulation is of primary concern in plant cell suspension culture, as its mechanisms can inhibit the production of key secondary metabolites over time.

DNA Methylation

DNA methylation is an epigenetic mechanism characterized by the addition of a methyl group to a cytosine residue, forming 5-methylcytosine. Methylation can significantly affect a cell's behavior, as it is engaged in the control of most genetic functions by means including gene silencing and parental imprinting [14]. It can also regulate expression of some genes by means of a methylated region avoiding the binding of a repressor protein [15]. As a result of its effects on gene control and regulation, DNA methylation can also impact secondary metabolite production. In *Taxus* cell cultures, it has been shown that there is a correlation between methylation level and the decline in long term secondary metabolite production [1]. A similar effect has been noted in transgenic birch *Betula platyphylla*, where a decrease in protein expression levels was associated with DNA methylation [16].

DNA can be methylated in two ways. The first, *de novo,* is the establishment of the methylation and is mediated by the RNA-directed DNA methylation pathway, where DNA methyltransferase 2 catalyzes methylation. Second, methylation is preserved and maintained after DNA replication by a series of enzymes [17]. Demethylation can naturally occur either passively or actively. In passive demethylation, a lack of DNA methyltransferase activity or shortage of a methyl donor post-replication can result in methylation maintenance failing. Active methylation can enzymatically occur [18].

Global DNA Methylation Engineering

Global DNA methylation engineering involves trying to reverse the DNA methylation that happens over time. In the case of plant cells, recent studies suggest that methylation is related to the biosynthesis of certain secondary metabolites [19]. This engineering represents a potential way to increase secondary metabolite production. One of the more commonly used methods is treatment with the demethylating agent 5-azacytidine. This compound is an analog of

cytosine and can be converted into a nucleotide and incorporated into the DNA. At this point, it bonds irreversibly with a DNA methyltransferase and leads to demethylation [19]. 5-azacytidine has already been proven effective as a demethylation agent, and for this reason was considered for this research [20, 21]. Another promising demethylating agent is zebularine. Though less studied than 5-azacytidine, it acts as a DNA methyltransferase inhibitor and has been shown to effectively re-express epigenetically silenced genes in low doses. For a DNA methyltransferase inhibitor, it is also quite stable, more so than 5-azacytidine [22].

These two treatments both affect the global DNA methylation, as opposed to targeted methylation engineering. This type of engineering involves methods through which specific genes can be targeted for demethylation, such as with dCas9 technology [23]. Through the lens of secondary metabolite production, however, global demethylation is a higher priority and a more viable option. Due to the complexity of the most synthesis pathways, targeted demethylation of a specific pathway via methylation engineering can be a difficult and time-consuming process. Creating a lower global methylation level through demethylating agents could potentially be a simpler alternative to control secondary metabolite production on a wider scale, similarly to how elicitation increases production. This finding would have several implications, and for the *Taxus* cell culture, it could be a viable way to increase paclitaxel production.

Research Aims

Overall, the primary objective of this research was to test the effectiveness of demethylating agents in increasing paclitaxel production. Prior research has shown that DNA methylation can impact the long-term yield of paclitaxel in *Taxus* cell cultures, thus it was hypothesized that the addition of demethylating agents could inhibit this epigenetic modification and prolong high

production levels. Both 5-azacytidine and zebularine were used as demethylating agents, and it was hypothesized that zebularine could potentially maintain high yields for longer, as it has been shown to be more stable in suspension cell culture. Lastly, additional secondary metabolites outside of the paclitaxel biosynthetic pathway were quantified, to test if the demethylating agents would generally upregulate secondary metabolism or if its effects are more specific to paclitaxel and its precursors.

VII. Methodology

Experimental Set-Up

An experimental timeline was established with two cell lines to explore the effects of demethylating agents on paclitaxel and other secondary metabolite production. The two cell lines subcultured from those maintained in lab were 48.82A.32 and P093XC. P093XC is an older cell line (approximately 25 years old), so it presumably has higher levels of DNA methylation, while 48.82A.32 is a newer cell line (approximately 4 years old). Each cell line was then maintained over a 3-week subculture cycle, and Table 1 below details the full timeline.

Table 1: *Description of experimental procedure performed. Experiment was performed over the course of 21 days, with samples taken on a weekly basis. Elicitation and demethylating agent* were *added on* Day *7.*

Day of Cycle	Procedure Performed
0	- P093XC and 48.82A.32 cell lines subcultured - All flasks sampled for paclitaxel and its precursors, additional secondary metabolites, and DNA methylation
7	- Flasks either elicited or mock-elicited, then demethylating agents added - All flasks sampled for paclitaxel and its precursors, additional secondary metabolites, and DNA methylation
14	- All flasks sampled for paclitaxel and its precursors, additional secondary metabolites, and DNA methylation
21	- All flasks sampled for paclitaxel and its precursors, additional secondary metabolites, and DNA methylation

On Day 0, the cells were subcultured, and sampled for baseline levels of paclitaxel, its precursors, and additional secondary metabolites. Samples were also taken to test for DNA methylation. The flasks were then elicited on Day 7 using either methyl jasmonate (MeJA+) for elicitation or a mock elicitor (MeJA-) to the flasks. For each group of either elicited or mock-elicited flasks, the demethylating agents zebularine or 5-azacytidine were added, and

concentrations of 100 µM and 200 µM were added. A diagram of this experimental flask set-up can be seen in Figure 2 below.

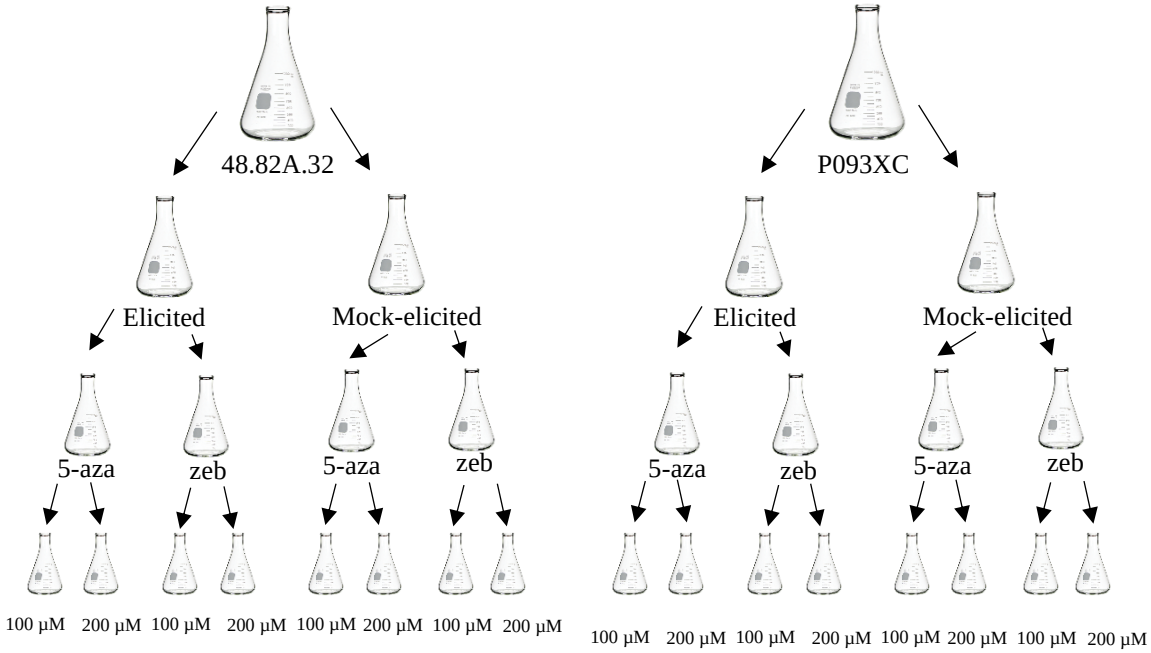

Figure 2: *Experimental flask set up. There was a total of 16 treatment groups along with an elicited and mock elicited control group for each cell line. 5-aza and zeb refer to 5-azacytidine and zebularine, respectively.*

A total of 60 flasks were maintained, with three biological replicates for each treatment. Note that for each group of elicited and mock-elicited cells, there was also a control flask where no demethylating agents were added. 4 maintenance flasks for each cell line were also kept and subcultured every two weeks, two for each cell line.

Subculturing Procedure

In a laminar flow hood, 30 mL of sterile antioxidant solution was added to 500 mL of sterile Gamborg B5 cell culture media. An additional 1.5 times the standard ratio of antioxidants, or 45mL, were added to the media to account for low cell density in both P093XC and 48.82A.32 cell suspension cultures. The cell culture media was then well mixed by swirling the bottle. A sterile 50 mL falcon tube was used to transfer 16 mL of the media and antioxidant

mixture into sterile 50 mL foam-capped Erlenmeyer flasks. 5 mL of well-mixed cell culture were transferred from P093XC and 48.82A.32 into their respective flasks using a stereological pipette. The flasks were then stored in an incubator at 125 RPM and 23˚C, and were not subcultured again during the remaining three week experiment. Any remaining media was stored in the refrigerator to be used again.

Maintenance flasks of each cell line were subcultured every two weeks in the same manner, except at a larger scale. 40 mL of media and antioxidant mixture and 10 mL of cell culture were transferred to account for the larger 125 mL flasks that the cell culture was maintained in. All other steps aside from this ratio and the two-week subculture cycle remained the same.

Elicitation with Methyl Jasmonate

Methyl jasmonate elicitation and mock elicitation stock solutions were prepared for addition to cell culture under the laminar flow hood. For the methyl jasmonate solution, 42.1 µL of 95% Sigma Aldrich methyl jasmonate was combined with 457.9 µL of ethanol and 500 µL nanopure water. The mock elicitation solution was prepared by combining 500 µL ethanol and 500 µL nanopure water. Each solution was then filter sterilized using an autoclaved 0.22 uM PVDF filter and 1.0 mL syringe, added into a 1.5 µL centrifuge tube.

Once prepared, the elicitation and mock elicitation standard solutions were added to each treatment flask on Day 7 of the experiment. To achieve the 200 µM elicitation, which is the standard concentration used in the Roberts laboratory for *Taxus* cell culture, 23.8 µL of each solution was sterilely added to their respective 50 mL flasks in a laminar flow hood. The flasks were sampled as described in Table 1, and then covered with foam caps and a layer of aluminum foil, to prevent cross-elicitation in the incubator. The flasks were returned to the incubator and

stored at 125 RPM and 23˚C. Methyl jasmonate and mock elicitation stock solutions were kept sterile and stored at 4˚C for future use.

Paclitaxel Extraction

Well-mixed samples of both media and cells were taken for paclitaxel quantification on Days 0-21. The samples were collected using a 1mL cut pipette tip and were transferred into a 1.5 mL centrifuge tube, then dried overnight in the evaporative centrifuge set to V-AQ. Dried cell matter was resuspended in 1000 µL acidified methanol (0.01% acetic acid in methanol), and a combination of sonication, vortexing, and breaking down with a spatula was used to ensure full separation of paclitaxel and cell matter. Once thoroughly resuspended, the samples were centrifuge for 20 minutes on the highest speed. 800 µL of supernatant was removed and transferred to a new 1.5 mL centrifuge tube. The supernatant was then dried in the evaporative centrifuge set to V-AL until completely try, approximately 1-2 hours. Samples could then be stored overnight or until needed in the -80˚C freezer.

To prepare the paclitaxel samples for the UPLC, the samples were resuspended in a ratio of 25:35:40 µL methanol/acetonitrile/water and sonicated for approximately 1 min after the addition of each component. After vortexing each sample, they were filtered using a 0.22 µM PVDF filter and 1.0 mL syringe into a low-volume UPLC vial, flushing the syringe with 1.0 mL methanol between each sample. The UPLC lid was crimped on completed samples and samples were flicked several times to remove air bubbles. The UPLC was then run immediately using the standard method for paclitaxel quantification routinely used in the Roberts laboratory.

DNA Extraction

Immediately after 1 mL well-mixed samples were taken on Days 0-21 of the experiment, a 1000 µL pipette was used to remove cell-suspension media from cell matter in each 1.5 mL

centrifuge tube. The 1000 µL pipette tip was also stacked with a 250 µL pipette tip on top of it to prevent cell matter from entering the pipette. The cell-suspension media was discarded in a waste container, and 1 mL of water was added to each sample. The samples were then centrifuged for 15 seconds, and the water was removed and discarded from the cell matter in the same manner as the cell-suspension media. Samples could then be stored overnight or until needed in the -80˚C freezer.

The samples were then extracted and hydrolyzed using the Macherey-Nagel Nucleospin Plant II Mini kit (with a 1-hour incubation time at 65C) and the Zymo Research DNA Degradase Plus™ procedures, respectively. However, unfortunately due to technical difficulties with the UPLC, these processed samples were not able to be analyzed to confirm the effectiveness of the demethylating agents. For this reason, the details of the remainder of this procedure will be omitted.

Secondary Metabolite Processing

Well-mixed samples of both media and cells were taken for additional secondary metabolite quantification, specifically flavonoids and phenolics, on Days 0-21. These samples were stored overnight or until needed in the -80˚C freezer.

To prepare samples for secondary metabolite quantification, the samples were thawed and dried overnight in the evaporative centrifuge set to V-AQ. They were then resuspended in 500 µL acidified methanol (0.01% acetic acid) and vortexed and sonicated in the same manner as described in the paclitaxel extraction procedure. Once thoroughly resuspended, the samples were centrifuged at the highest speed for 5 min.

Different preparation methods were required for the flavonoid and phenolics assays, and each preparation method was performed on all samples. For the flavonoids assay, catechin

standards in concentrations of 1.0 mg/mL, 0.8 mg/mL, 0.6 mg/mL, 0.4 mg/mL, 0.2 mg/mL, 0.1 mg/mL, and 0.0 mg/mL were prepared. Then, 25 µl of each sample and standard were added to plate well, with 3 plate wells per sample. 50 µl of deionized water, 75 µl $NaNO_2$ (6 g/L), and 75 µl $AlCl_3$ (22 g/L $AlCl_3 \cdot 6H_2O$) were added to each plate well and allowed to incubate for two minutes. Following the incubation, 75 µl 0.8M NaOH was added to each well, and then the plate was read on the plate reader at 490 nm.

For the phenolics assay, gallic acid standards in concentrations of 0.20 mg/mL, 0.15 mg/mL, 0.10 mg/mL, 0.075 mg/mL, 0.05 mg/mL, 0.025 mg/mL, 0.0 mg/mL were prepared. Then, in separate 1.5 mL centrifuge tubes, 60 µL of samples/standards, 120 µL 0.2N FC Reagent, and 480 µL of 700 mm sodium carbonate were added. This new mixture was incubated for 10 minutes and centrifuged at the highest speed for 1 minute. 200 µL of the supernatant was transfer to the plate wells, with 3 plate wells per sample. The plate was read on the plate reader at 750 nm.

VIII. Results and Discussion

Paclitaxel Quantification

After quantifying paclitaxel using the UPLC, the concentrations on each day for all treatments were recorded and are represented graphically below in Figure 3.

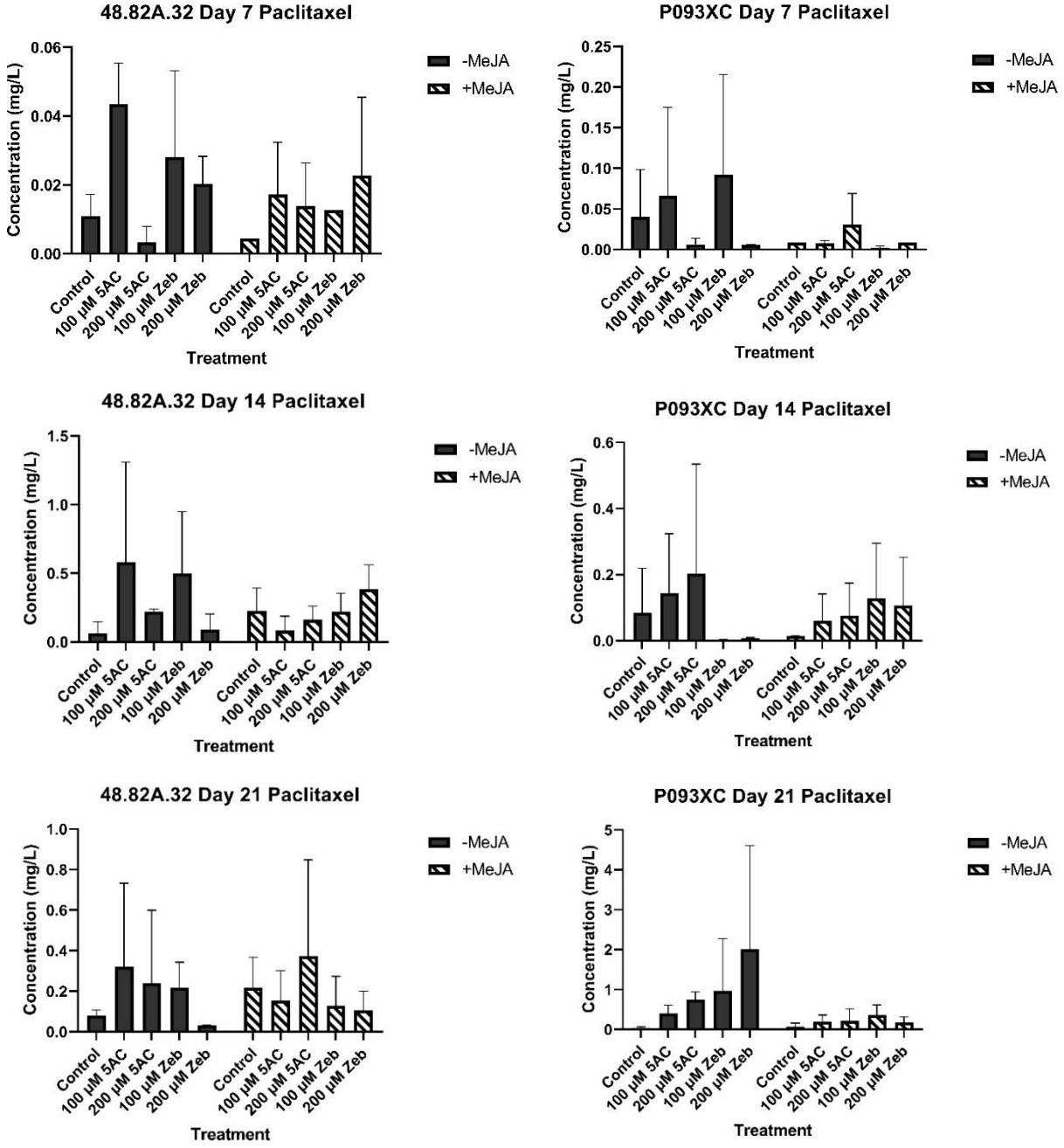

Figure 3: *UPLC data for paclitaxel quantification. Concentration was based on a standard curve calculated each day the UPLC was run. Unelicited samples are depicted by the solid bars, and elicited samples are depicted by the striped bars.*

Between days, paclitaxel concentration appears to significantly increase between Day 7 and Day 14 for both cell lines, and then remain mostly consistent between Day 14 and Day 21. The statistical significance of this finding was also confirmed via Two-way ANOVA analysis for the 48.82A.32 cells, but not for P093XC. P093XC concentration levels, particularly in the unelicited flasks, likely had standards of deviation that were too high for there to be statistical significance. The fact that the largest increase across days corresponded to when treatment was added, while not statistically significant in both cell lines, could indicate that the demethylating agents were successful in increasing the paclitaxel production. However, since the control flasks also increased during that time and there was no statistical significance between the treatments of the control, it is possible that the paclitaxel levels increased naturally during that point in the cell cycle.

A number of the findings from this data were unexpected based on what was hypothesized from prior research. Elicitation is confirmed to increase paclitaxel production, and is widely used in industry for this purpose, yet these results do not demonstrate this. For both 48.82A.32 and P093XC, the unelicited flasks appear to have a generally higher concentration than the control flasks for Days 14 and 21. Even though there was found to be no statistical significance between elicited and mock-elicited flasks across both cell lines on each day they were sampled, that is still an unexpected occurrence. A possible explanation for the lack of significance between elicitation and mock elicitation is that fresh methyl jasmonate was not used on Day 7 of the experiment. The age of the solution could potentially make the elicitor less effective at inducing a stress response in the cells, and consequentially produce less paclitaxel. However, assuming that elicitation was able to occur regardless of freshness, a possible

explanation is that elicitation is more effective on less methylated cell lines. Since the P093XC cell line is older than 48.82A.32, and thus more methylated, it is possible that there was too much DNA methylation for elicitation to be effective. This could also potentially explain the lack of difference in the 48.82A.32, as even though it is one of the newer cell lines within the lab, it has still likely accrued some degree of methylation over time. Lastly, it is possible that there was not a significantly higher concentration of paclitaxel in the elicited cells due to an interaction between the demethylating agents and the elicitation solution. However, additional experiments would have to be run to confirm this possibility.

Additionally, the lack of a significant difference between treatment and controls for both cell lines was unexpected when compared to their graphical depictions. There visually appears to be a positive correlation between treatment and paclitaxel production, but this was not backed by statistical analysis. This is likely due to the high standards of deviations across the data. There are a few reasons why there is so much deviation. First, while sampling for each treatment group in triplicate was the intent, low cell density due to the large number of samples taken from a relatively small total volume (20 mL) made it impossible to collect enough samples for some of the flasks. Despite efforts to increase cell density by using smaller flasks and a higher concentration of antioxidants in media, taking too many samples would have resulted in using all remaining cell matter before the final day of sample collection. Thus, some values are based only from two samples. Second, technical problems with the UPLC resulted in some samples being prepared, and then having to sit for nearly a week before being run. This could impact the results, since the paclitaxel may have degraded into its precursors after sitting for too long in the solvent used when preparing the samples. This could skew both the paclitaxel data and the data for its

precursors. Furthermore, the statistical insignificance between treatment and control, despite the visuals showing a difference, makes it so treatment cannot be claimed to be effective.

Lastly, another particularly unexpected finding was the lack of a significantly different effect between 5-azacytidine and zebularine. 5-azacytidine as a demethylating agent has been studied more than zebularine and is known to quickly degrade in cell suspension culture. Zebularine, however, is much more stable, which was the motivation to study it alongside 5-azacytidine. Theoretically, zebularine should then be active for longer in the cell and should have a larger effect, particularly after Day 14 when the treatments were added, but this was not found to be the case. Only in P093XC unelicited cells was there any significant difference between concentrations in Day 14 and Day 21, particularly in 200 µM zebularine, but this was not enough to draw conclusive evidence.

Quantification of Paclitaxel Precursors

When processing the paclitaxel samples on the UPLC, the concentrations of its precursors, 10-deacetylbaccatin (10-DAB) and baccatin, were also analyzed in case there were any noticeable changes at other stages of paclitaxel metabolism. For 10-DAB, the first precursor to paclitaxel, there was found both visually and statistically to be no difference between treatments and elicitation within any each sampled. A graphical representation of this is provided below in Figure 4. This could indicate that DNA methylation does not regulate 10-DAB production. Since 10-DAB is the first precursor, if methylation specifically regulates later in the pathway, it would be unaffected.

For both elicited and mock elicited 48.82A.32, there is no significant difference between days sampled as well. There was, however, a significant difference between Day 7 and Day 14 for both elicited and unelicited P093XC. For these cells, the concentration notably decreased

from between these days, as seen in Figure 4. There was no significant difference between Day 14 and Day 21 for either, so the general decrease over time in both cell lines is likely due to more of the 10-DAB being converted into later compounds in the paclitaxel biosynthetic pathway, such as baccatin and paclitaxel, rather than a specific interaction with the demethylating agents. Additional graphs for Day 21 can be found in Appendix A

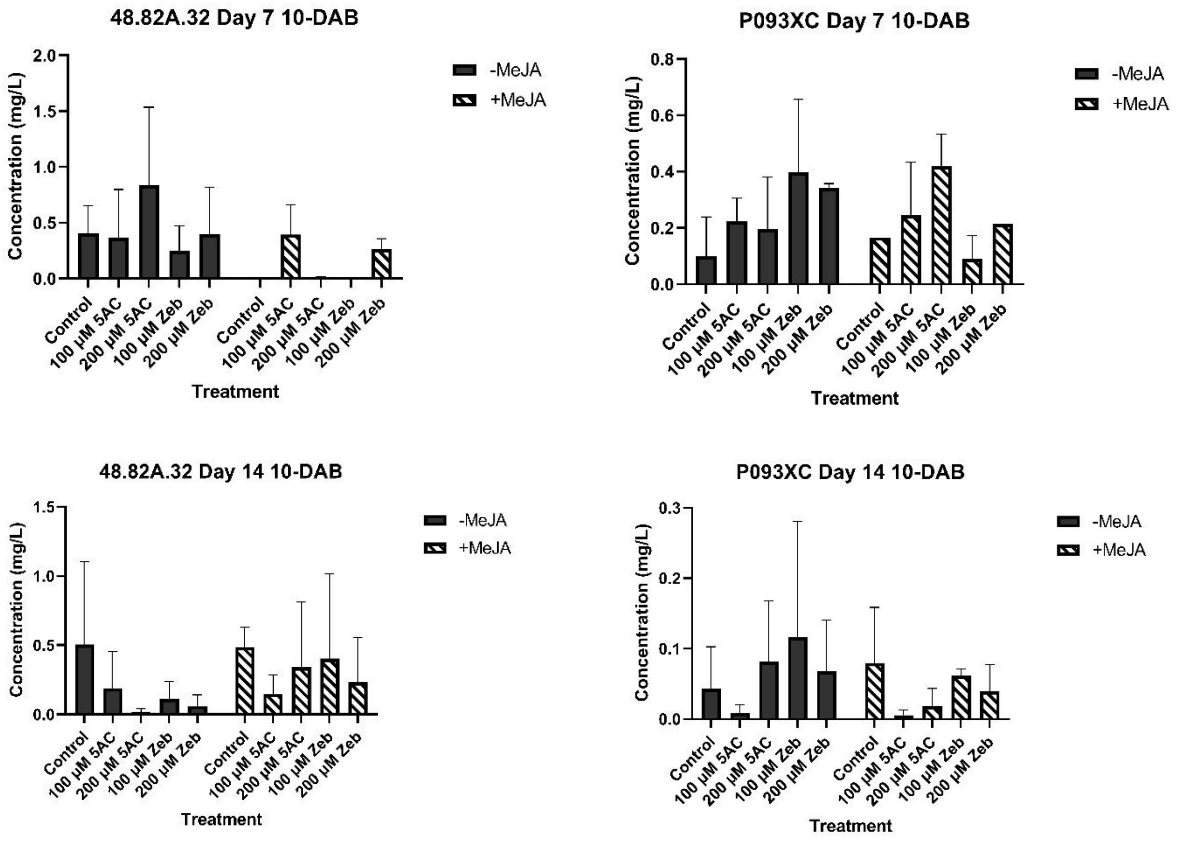

Figure 4: *UPLC-based quantification of 10-DAB. Concentration was based on a standard curve calculated each day the UPLC was run. Unelicited samples are depicted by the solid bars, and elicited samples are depicted by the striped bars.*

Baccatin is the second precursor to paclitaxel. The most notable finding from this data is the concentration increase between Day 14 and Day 21, where as shown in Figure 5, the concentrations of paclitaxel in both 48.82A.32 and the P093XC cell lines saw a 10-fold increase in some treatments. While the 48.82A.32 is less significant of a change, the amount of baccatin

seems too high to ignore, especially when compared with the P093XC, which did experience statistically significant change. A potential reason for the spike is if the paclitaxel production pathway is bottlenecked at baccatin. It would make sense, with the previously noted small concentrations, that most of the 10-DAB became baccatin, but if something was preventing baccatin from being converted to paclitaxel, there would naturally be an increased concentration. However, since it occurred to a higher extent in the unelicited cells, this theory may not be plausible, as the constant paclitaxel levels between elicited and unelicited would indicate that these levels should also be similar.

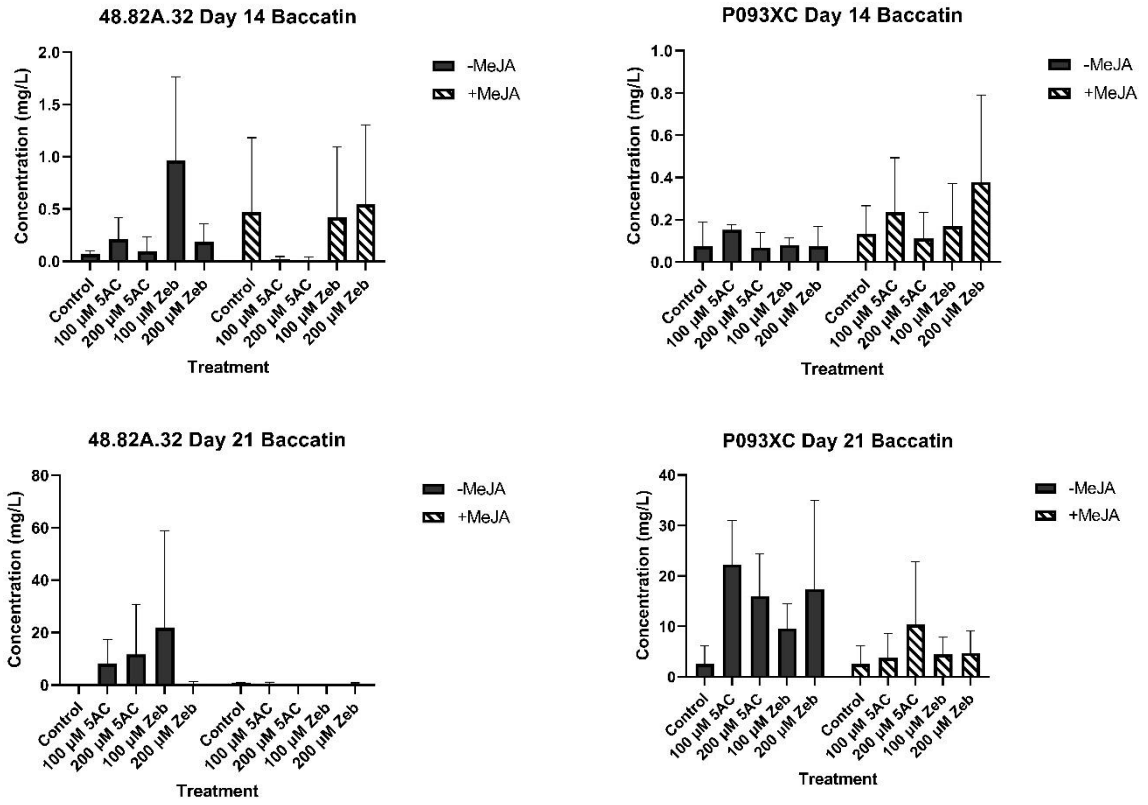

Figure 5: *UPLC quantification of baccatin. Concentration was based on a standard curve calculated each day the UPLC was run. Unelicited samples are depicted by the solid bars, and elicited samples are depicted by the striped bars.*

For 48.82A.32 Baccatin levels, there was no significant or visual difference between treatments and elicitation. For P093XC, there is a difference between elicited and non-elicited

cells on Day 21, with non-elicited cells being higher. This is visually confirmed. In terms of days, there was no significant change in concentration in 48.82A.32 flasks throughout the course of the experiment. Visually, there was a notable change in the Day 21 elicited flasks, with three of them being significantly higher. For P093XC, there was a significant change between Day 14 and Day 21 for both elicited and unelicited cells, which is confirmed visually. As with 10-DAB, there was no consistent difference between treatment flasks and control flasks, also potentially indicating that the creation of baccatin is not regulated by DNA methylation.

Secondary Metabolite Quantification: Flavonoids and Phenolics

Assays for both flavonoids and phenolics were also performed to determine whether demethylating agents generally upregulate secondary metabolism, or if their effects are more specific to paclitaxel. First, the flavonoid readings were analyzed. Results were normalized to the mock elicited control and presented in terms of fold. Across both cell lines, it was found that flavonoid concentration stayed visually consistent despite treatment, and this was also confirmed by statistical analysis.

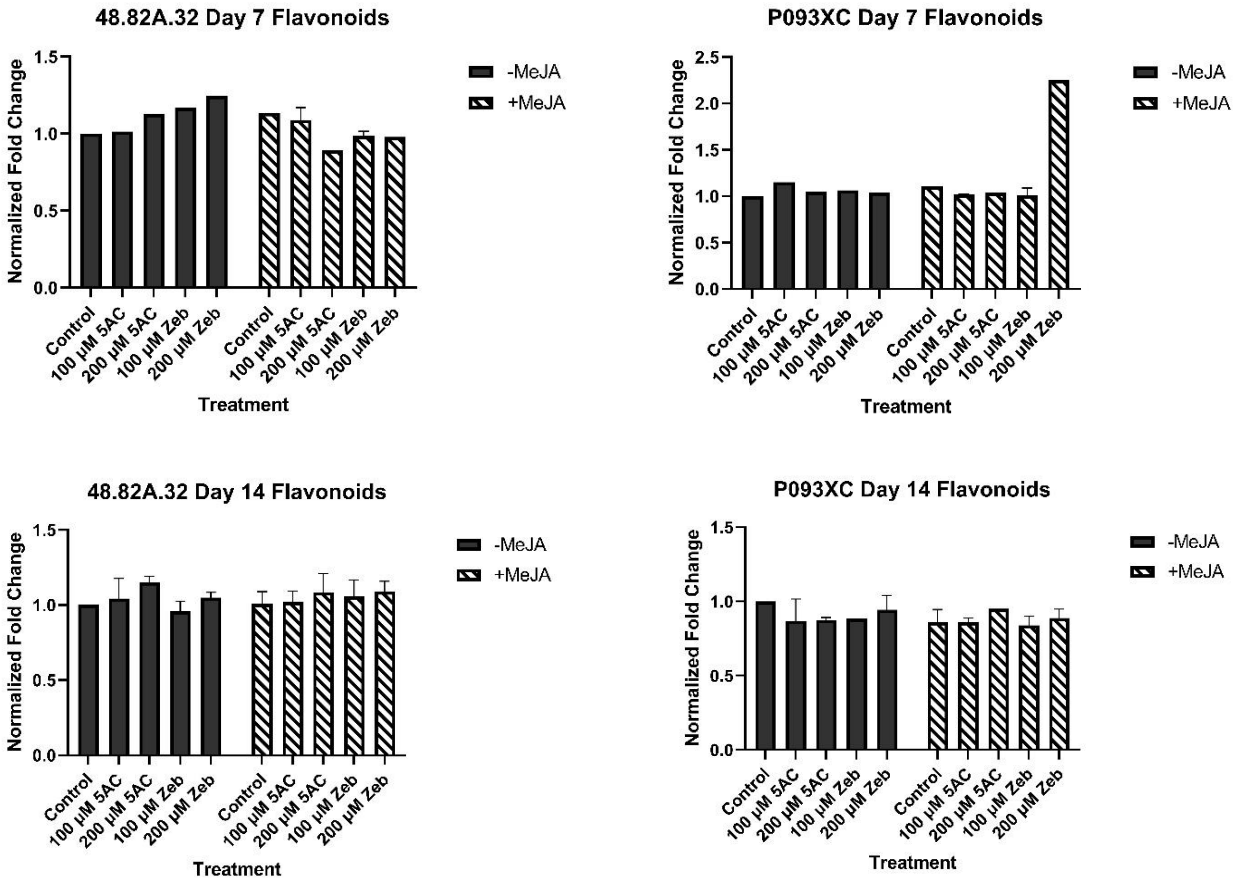

Figure 6: *Graphical representation of flavonoid data obtained from a plate reader assay. Data was normalized to the respective mock elicited control flask for each group and presented in terms of fold. Unelicited samples are depicted by the solid bars, and elicited samples are depicted by the striped bars.*

Furthermore, there was no difference between days and treatments for both cell lines, spare the outlier of the 200µM elicited zebularine for Day 7 P093XC, shown in Figure 6. These results could indicate that the flavonoid production pathway is not governed by DNA methylation and is thus entirely unaffected by demethylation treatment. However, it could also be the result of too low of concentrations being used, and not enough DNA methylation being reversed to have a notable effect.

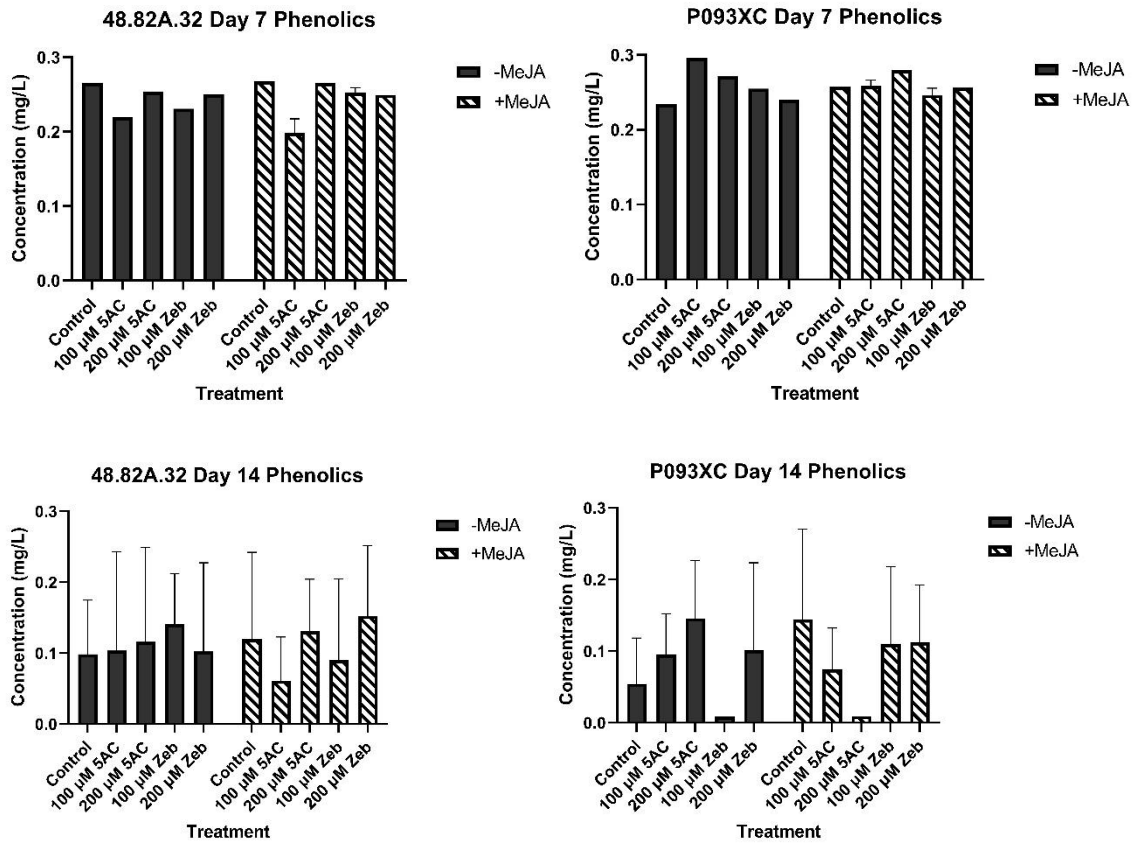

Figure 7: *Graphical representation of phenolic data obtained from a plate reader assay. Concentration was calculated using a standard curve. Unelicited samples are depicted by the solid bars, and elicited samples are depicted by the striped bars.*

Phenolic results largely mimic the flavonoid results, with no significant difference between treatments and elicitation within each day sampled. The results do vary significantly, however, between Day 7 and Day 14 for both the unelicited P093XC and the elicited 48.82A.32. It is interesting to note is that there is no major difference in any of the individual flasks across the time period, but the mean across each set is larger. Furthermore, the Day 14 data also has a very large standard of deviation that is lacking in the Day 7 and Day 21 data. Again, as in the flavonoids, the results could indicate that phenolic production pathway is unaffected by DNA methylation, or that there was not enough demethylation that occurred to make a difference.

IX. Conclusions

This MQP looked to investigate the effects of demethylating agents 5-azacytidine and zebularine on the production of various secondary metabolites in the *Taxus* cell. Specifically, investigation was done of paclitaxel, its precursors, flavonoids, and phenolics. Due to a relatively small sample size, there was a large deviation with the results that caused most of them to be statistically insignificant, even if visuals suggested otherwise. That being said, data suggested that there could be a correlation between treatment and paclitaxel production, but the large standards of deviations make it so a definitive correlation could not be claimed. Treatment was found to have no effect on the precursors 10-DAB and the statistical differences noted between Days 14 and Day 21 in baccatin cannot be conclusively tied to the demethylating agents. There was also no effect on flavonoid and phenolic production, which suggests that DNA methylation likely specifically targets the paclitaxel production pathway, as opposed to regulating secondary metabolite production overall. Furthermore, there was found to be no difference in effect between 5-azacytidine and zebularine, despite the expected increased stability in zebularine. Elicitation was also found to have no significant effect on any of the secondary metabolite production, which is contradictory to what was expected from industry practices, so this interaction should be investigated further. Overall, demethylating treatment remains a promising method to increase paclitaxel production but will likely have no effect on other secondary metabolites.

X. Future Work

Throughout the course of this MQP, there were several areas identified that would benefit from further research. The first would be to investigate the optimal time for treatment to be administered. In the experiments described above, all treatments were added on Day 7. Knowing how the paclitaxel levels are affected by variance in treatment time could provide further insight into how exactly the demethylating agents affect the cells. The most notable observed change in concentration from MQP experiments – between day 7 and day 14 – corresponded to when treatment was administered, and it could be important to know if that would occur if administration time varied.

Investigation into how frequently treatment needs to be administered should also be done. Since there was a notable fall-off in concentration after day 14, it would be important to know whether repeated treatment would maintain concentration levels. Additionally, since we know that 5-azacytidine degrades relatively rapidly in solution and could be short lasting, this leads to the potential for continuous treatment over time, which also should be investigated.

Furthermore, future research should be done into the interaction that occurs when using methyl jasmonate and a demethylating agent simultaneously. The results obtained in this MQP were in opposition to what was expected, as even in the newer cell line elicited flasks did not have a higher overall concentration.

Additionally, future research should study the effects of both demethylating agents on cell viability. Since the treatments are relatively harsh, treatment should correspond to a decrease in cell viability, yet this was not observed. Research then should determine the maximum concentration of treatment before cells become completely non-viable, and whether or not that increased concentration corresponds with even more paclitaxel production.

The effects of treatment on global methylation levels of cells should also be analyzed. While this was originally a goal of this MQP, time constraints and technical problems forced it to be cut. This MQP was going to measure levels via UPLC, measuring the relative peaks between methylated and unmethylated nucleotides. A more accurate method, however, could be the use of RT-qPCR to measure how the demethylating treatments affect expression of paclitaxel biosynthetic pathway genes. Performing this along with the use of bisulfite sequencing to measure DNA methylation of those same genes would provide an exact sequence of methylated and unmethylated nucleotides for those genes. Global methylation levels would provide further insight into the effectiveness of the treatments. The greater paclitaxel concentration arising from treatment would indicate the treatment successfully demethylates some of the DNA, but confirmation would ensure a more definitive correlation. Additionally, this analysis would help with determining how frequently treatments would be administered, and whether or not continuous treatment over a longer period of time would be successful in reversing some of the methylation that has occurred over time. It would also provide clearer insight into how comparatively effective 5-azacytidine and zebularine are as demethylating agents.

XI. Potential for Scale-Up

Upon successful completion of the additional experiments and future work suggested, there is potential for the use of demethylating agents at an industrial scale. Currently, the largest global supplier of paclitaxel is Phyton Biotech, which provides Bristol-Myers Squibb with the paclitaxel necessary to produce the widely used anti-cancer treatment, Taxol® [24]. It is hypothesized that Phyton's Plant Cell Fermentation (PCF) method that is used to supply high yields of paclitaxel could easily be modified to include demethylating agents.

The PCF process is designed to address the lack of plant cell matter necessary to extract paclitaxel. Figure 8 below shows a schematic of the steps leading up to the scale up process.

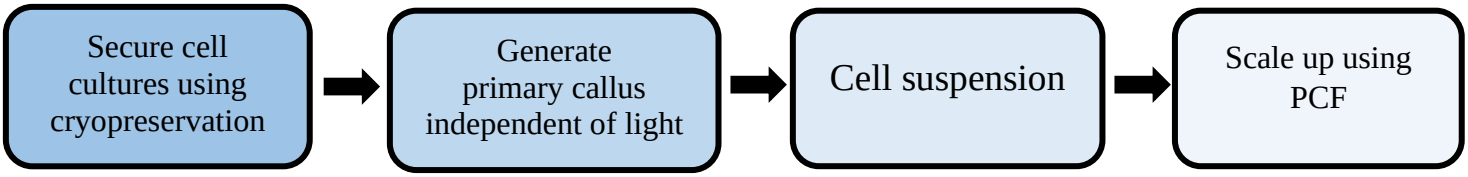

Figure 8: *This schematic shows the step-by-step process of the Plant Cell Culture Fermentation (PCF) process. The unused cell cultures are stored at temperatures of -200° C or less, and then enter the process through careful thawing and generation of the primary callus independent of light. They are then suspended and incrementally scaled-up to produce high yields [25].*

These alterations in media formulations and environmental conditions throughout the process encourage the cells to produce paclitaxel in much shorter time frames than previously reported [26]. Specifically, many different enhancement agents, such as a variety of inhibitors, elicitors (including methyl jasmonate), and stimulants are used in the cell suspension media. From the findings in this MQP, it is hypothesized that demethylating agents could be seamlessly added during this step of the PCF process to increase yields of paclitaxel. Then, compared to its stability in typical laboratory conditions, the demethylating agents would likely display no adverse effects when further cultivated in the batch process [26]. Further research must be done

to confirm the benefits and potential effects of demethylating agents in *Taxus* cell culture, however the ease of its addition to a widely accepted production process makes it a viable option for industrial scale-up.

XII. References

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XIII. Appendices

Appendix A: Additional Figures

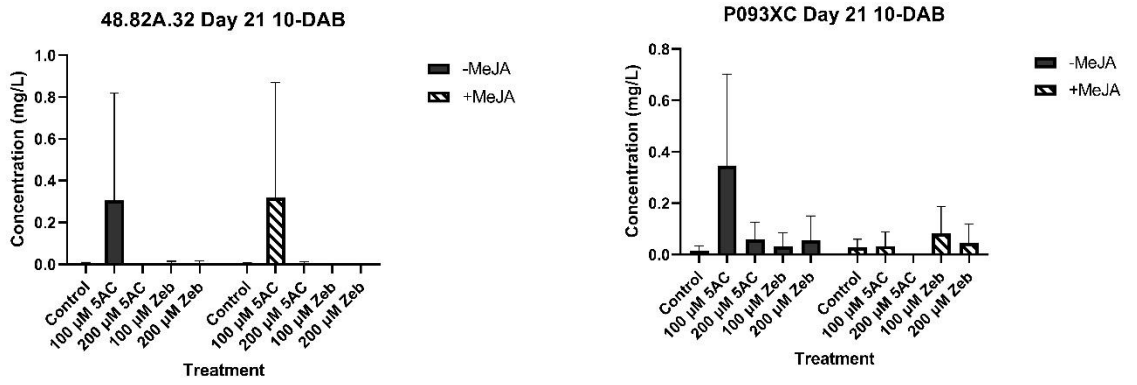

Figure 9: *UPLC-based quantification of 10-DAB on Day 21. Concentration was based on a standard curve calculated. Unelicited samples are depicted by the solid bars, and elicited samples are depicted by the striped bars.*

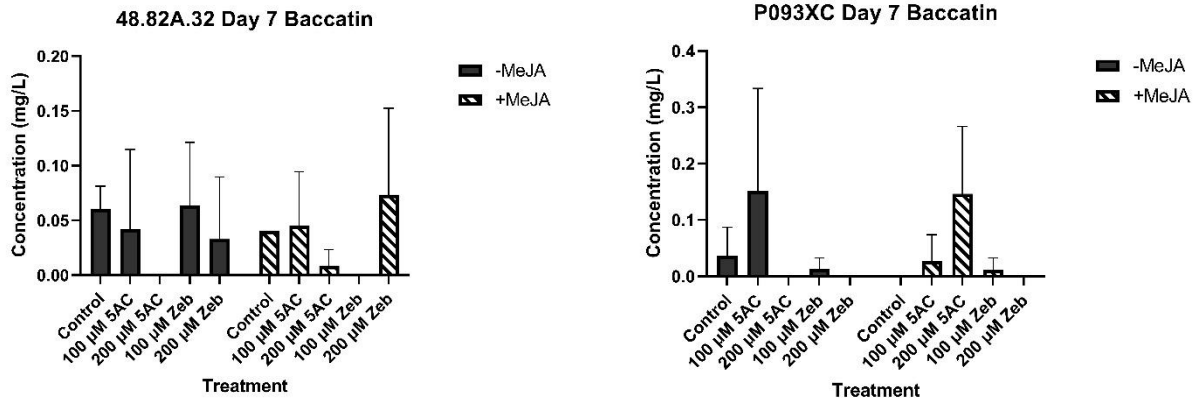

Figure 10: *UPLC-based quantification of baccatin on Day 7. Concentration was based on a standard curve calculated each day the UPLC was run. Unelicited samples are depicted by the solid bars, and elicited samples are depicted by the striped bars.*

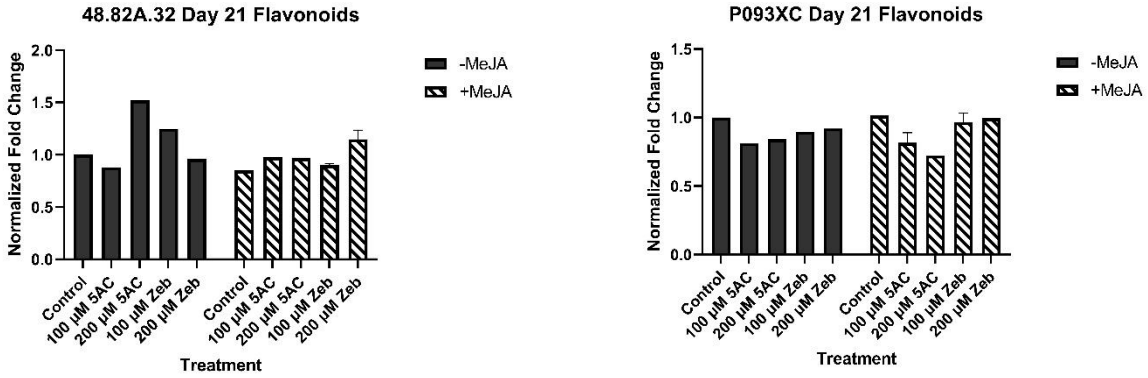

Figure 11: *Graphical representation of flavonoid data from Day 21 obtained from a plate reader assay. Data was normalized to the respective mock elicited control flask for each group and presented in terms of fold. Unelicited samples are depicted by the solid bars, and elicited samples are depicted by the striped bars.*

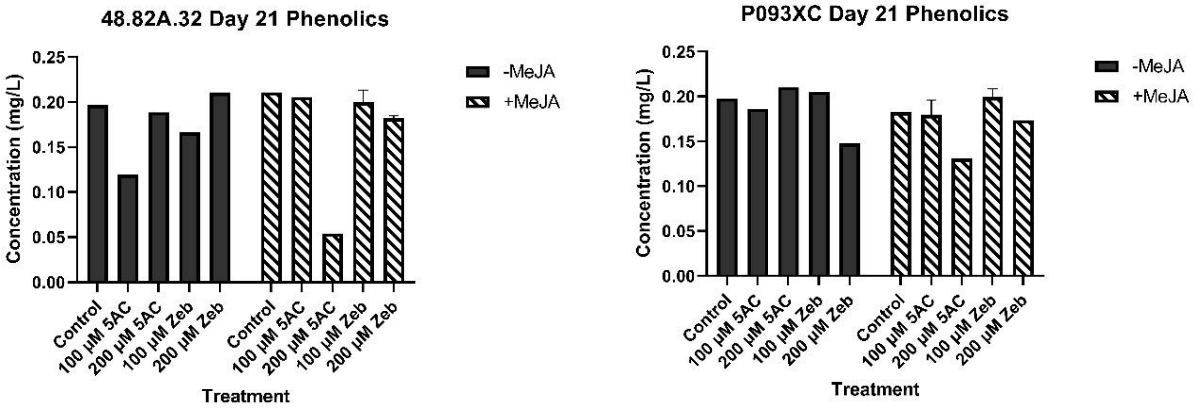

Figure 12: *Graphical representation of phenolic data from Day **blank** obtained from a plate reader assay. Concentration was calculated using a standard curve. Unelicited samples are depicted by the solid bars, and elicited samples are depicted by the striped bars.*

Appendix B: Raw Data

B.1 UPLC Data

B.1.1 Calibration Curves:

Table 2: *Concentrations used to determine the standard paclitaxel curves for samples 1-48.*

Concentration (µg/L)	10-DAB Area	Baccatin Area	Paclitaxel Area

5	77381	86954	144434
1	18436	19667	35897
0.1	4221	5488	10576

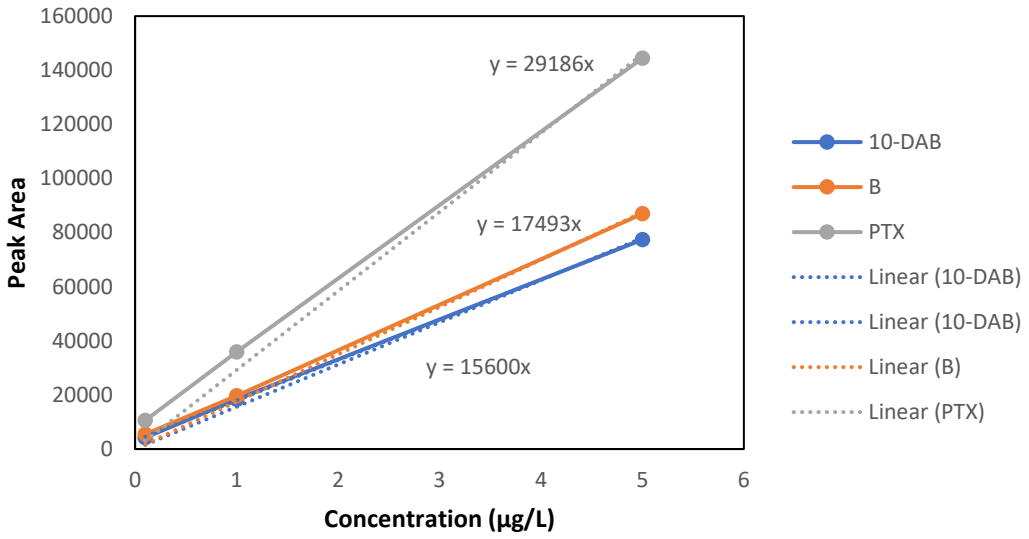

Figure 13: *Standard curves for samples 1-48 for 10-DAB, baccatin (B) and paclitaxel (PTX). The equation is above each line.*

Table 3: *Concentrations used to determine the standard paclitaxel curves for sample 121-144.*

Concentration (µg/L)	10-DAB Area	Baccatin Area	Paclitaxel Area
10	9139	8734	8003
5	4712	4433	4099
2	1848	1756	1610
1	957	930	806
0.5	473	431	405
0.2	196	158	195
0.1	79	72	40

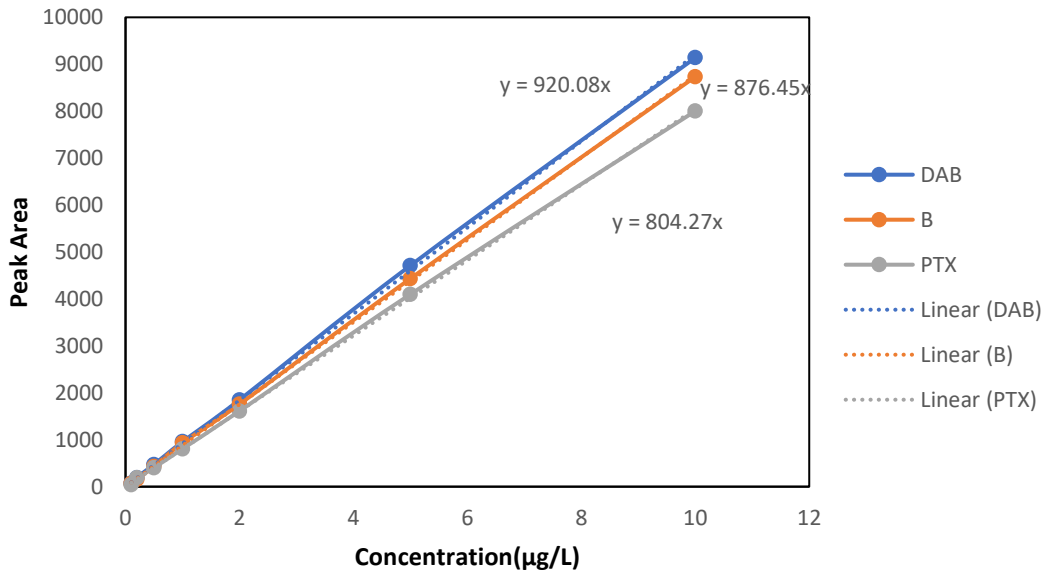

Figure 14: *Standard curves for samples 121-144 for 10-DAB, baccatin (B) and paclitaxel (PTX)..*

Table 4: *Concentrations used to determine the standard paclitaxel curves for sample 73-96.*

Concentration (µg/L)	10-DAB Area	Baccatin Area	Paclitaxel Area
10	8357	8142	7628
5	4048	4042	3805
2	1796	1679	1587
1	867	997	844
0.5	340	456	406
0.2	151	148	130
0.1	54	38	108

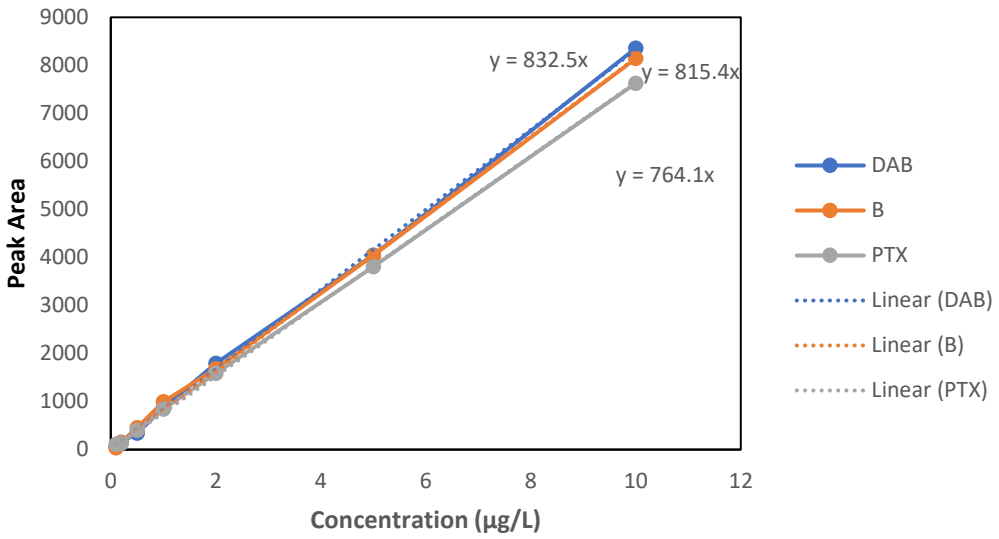

Figure 15: *Standard curves for samples 73-96 for 10-DAB, baccatin (B) and paclitaxel (PTX).*

B.1.2 Day 7 Data:

Table 5: *Concentrations of 10-DAB, Baccatin, and Paclitaxel for samples 1-48. Concentration calculations were performed using a standard curve.*

	Specifications			**Concentration (µg/L)**		
Sample #	Cell Line	Elicitation	Treatment	10-DAB	Baccatin	Paclitaxel
1	P093XC	no	100 aza	0.291602564	0.353741497	0.191735764
2	48.82A.32	no	200 aza	1.332307692	0	0
3	P093XC	no	100 zeb	0.580705128	0	0.179435346
4	P093XC	yes	100 zeb	0	0	0
5	48.82A.32	yes	200 aza	0	0	0
6	P093XC	no	200 aza	0.113910256	0	0
7	48.82A.32	no	None	0.266923077	0.044074773	0.012266155
8	P093XC	yes	100 aza	0.141410256	0	0.004077297
9	P093XC	yes	200 aza	0.29224359	0.071800149	0.014801617
10	P093XC	no	None	0.197948718	0.072085977	0.081443158
11	48.82A.32	no	200 zeb	0	0	0.013568149
12	48.82A.32	yes	200 aza	0.020705128	0.025381581	0.017508394
13	48.82A.32	no	100 aza	0	0	0.03080244

14	P093XC	yes	200 zeb	0.214551282	0	0.008428699
15	P093XC	yes	100 aza	0.136346154	0	0.010553005
16	48.82A.32	yes	200 aza	0	0	0.024292469
17	P093XC	no	200 zeb	0.353076923	0.000743154	0.004625505
18	P093XC	no	none	0	0	0
19	P093XC	no	100 aza	0.130064103	0	0
20	P093XC	no	200 zeb	0.332371795	0	0.006304393
21	48.82A.32	no	100 zeb	0	0.077688218	0.035359419
22	P093XC	yes	none	0.166538462	0	0.008462962
23	48.82A.32	yes	none	0	0.040187504	0.00445419
25	P093XC	yes	200 aza	0.513141026	0.28445664	0.074487768
26	48.82A.32	no	200 zeb	0.834230769	0.098610873	0.018536285
27	48.82A.32	no	200 aza	0.346794872	0	0.00661276
28	48.82A.32	yes	100 aza	0.507628205	0.097753387	0.008257384
29	48.82A.32	yes	200 zeb	0.259358974	0.062996627	0.011341054
30	P093XC	no	200 aza	0.407884615	0	0.004591242
31	48.82A.32	yes	200 zeb	0.360705128	0.157091408	0.048893305
32	48.82A.32	no	none	0.69224359	0.083747785	0.003871719
33	48.82A.32	no	100 zeb	0.329423077	0.112787972	0.04872199
34	48.82A.32	no	100 aza	0.841602564	0.12605042	0.054580963
35	P093XC	no	100 zeb	0.21474359	0.026696393	0.004899609
36	48.82A.32	no	100 aza	0.255897436	0.000743154	0.045090112
37	48.82A.32	yes	100 aza	0.090641026	0	0.008908381
38	48.82A.32	no	none	0.252884615	0.053964443	0.016514767
39	P093XC	no	200 aza	0.068653846	0	0.01449325
40	P093XC	yes	100 zeb	0.103846154	0.03595724	0.004933872
41	P093XC	no	100 aza	0.248525641	0.099525524	0.005790447
42	P093XC	yes	100 aza	0.461987179	0.081175327	0.008223121
43	P093XC	yes	200 aza	0.45275641	0.082432973	0.003152196
44	48.82A.32	no	100 zeb	0.419230769	0	0
45	48.82A.32	yes	100 aza	0.586474359	0.038072372	0.034708422

46	P093XC	yes	100 zeb	0.164487179	0	0.001815939
47	48.82A.32	yes	200 zeb	0.178846154	0	0.008051806
48	48.82A.32	no	200 zeb	0.358333333	0	0.029089289

B.1.3 Day 14 Data:

Table 6: *Concentrations of 10-DAB, Baccatin, and Paclitaxel for samples 74-120. Concentration calculations were performed using a standard curve.*

Specifications				**Concentration (µg/L)**		
Sample #	Cell Line	Elicitation	Treatment	10-DAB	Baccatin	Paclitaxel
74	48.82A.32	no	200 aza	0	0	0.202853
75	48.82A.32	yes	200 zeb	0.46006006	1.080451	0.256511
76	P093XC	no	200 aza	0.17297297	6.323277	0.586311
77	48.82A.32	yes	100 zeb	0.09009009	1.199411	0.338961
78	48.82A.32	yes	100 zeb	0	0.017169	0.246041
79	48.82A.32	yes	none	0.33153153	1.292617	0.243424
80	48.82A.32	no	200 aza	0.03603604	0.028207	0.21594
81	48.82A.32	yes	200 zeb	0	0.009811	0.509096
82	48.82A.32	no	100 zeb	0.24984985	0.952907	0.193692
83	P093XC	yes	100 zeb	0.06846847	0.028207	0.011779
84	48.82A.32	no	100 zeb	0.08048048	1.77091	0.281377
85	P093XC	yes	100 zeb	0.05525526	0.311504	0.246041
86	P093XC	no	100 aza	0.01681682	0.169242	0.270907
87	48.82A.32	yes	100 aza	0.24384384	0	0.157048
88	P093XC	no	none	0	0.017169	0.239497
89	P093XC	yes	100 aza	0	0.4182	0.117786
90	48.82A.32	no	200 zeb	0.11651652	0.307824	0.170135
91	48.82A.32	no	100 aza	0	0.355654	1.096715
92	48.82A.32	no	100 zeb	0	0.165563	1.018191
93	P093XC	yes	200 aza	0.03603604	0.025754	0.145269
94	48.82A.32	no	none	0.01081081	0.099338	0.159665
95	48.82A.32	yes	200 aza	0.00600601	0	0.231645

96	P093XC	yes	200 zeb	0.01201201	0.66961	0.209397
97	P093XC	yes	200 zeb	0.06599623	0.087956	0.00428
98	P093XC	yes	100 aza	0.01064813	0.054992	0.003407
99	48.82A.32	no	200 zeb	0.0032536	0.064201	0.008012
100	P093XC	yes	none	0.13543092	0.039838	0.014989
101	48.82A.32	no	none	0.3299072	0.072897	0.016976
102	P093XC	no	none	0.01793175	0	0.009066
103	P093XC	no	200 aza	0.07032203	0.118554	0.007139
104	48.82A.32	no	200 aza	3.8941842	0.252422	0.241907
105	48.82A.32	yes	none	0.61740674	0.042683	0.055391
106	48.82A.32	no	none	1.16966762	0.033667	0.005456
107	48.82A.32	no	100 aza	0.37530965	0.065128	0.066567
108	P093XC	no	200 zeb	0.15144009	0.043418	0.00931
109	P093XC	no	200 zeb	0.01848634	0.180932	0.006612
110	P093XC	no	100 aza	0	0.135051	0.01653
111	P093XC	no	100 zeb	0.23270603	0.104294	0.003144
112	P093XC	no	100 zeb	0	0.058605	0.001582
113	P093XC	no	none	0.11102895	0.20798	0.00789
114	48.82A.32	yes	100 zeb	1.10999371	0.041053	0.072631
115	48.82A.32	yes	none	0.50075794	0.06986	0.383945
116	P093XC	no	200 aza	0	0.017425	0.01296
117	P093XC	no	200 zeb	0.03427367	0	0.008498
118	48.82A.32	yes	100 aza	0.05057862	0.037919	0.005902
119	P093XC	yes	none	0.02310792	0.227739	0.014319
120	P093XC	yes	200 aza	0	0.200435	0.005902

B.1.4 Day 21 Data:

Table 7: *Concentrations of 10-DAB, Baccatin, and Paclitaxel for samples 121-177. Concentration calculations were performed using a standard curve.*

Specifications	Concentration (µg/L)

Sample #	Cell Line	Elicitation	Treatment	10-DAB	Baccatin	Paclitaxel
121	P093XC	no	200 aza	0	25.70141	0.636602
122	48.82A.32	yes	200 zeb	0	0.888813	0
123	48.82A.32	no	100 aza	0.89666116	6.241086	9.970532
124	P093XC	yes	100 aza	0.09673072	0	0.344412
125	48.82A.32	no	200 aza	0	33.62086	0.655253
126	P093XC	no	100 aza	0.75645596	138.2098	0.41777
127	48.82A.32	yes	100 aza	0	0.992641	0.293434
128	P093XC	yes	100 aza	0	9.156255	0.034814
129	48.82A.32	yes	200 aza	0	0	0.709961
130	P093XC	yes	200 zeb	0.13042344	8.470535	0.330735
131	48.82A.32	no	200 zeb	0	1.005191	0.031084
132	48.82A.32	yes	100 zeb	0	0	0.231266
133	P093XC	yes	100 zeb	0.04238762	7.873809	0.236239
134	48.82A.32	yes	100 zeb	0	0.08215	0.026111
135	48.82A.32	yes	100 aza	0.95426485	0.739346	0.170341
136	48.82A.32	no	100 aza	0	18.26801	0.612978
137	P093XC	no	100 aza	0.12498913	16.1093	0.604275
138	P093XC	no	100 zeb	0	11.54772	0.103199
139	P093XC	no	100 aza	0.15324754	28.46255	0.200182
140	48.82A.32	no	100 zeb	0	64.63232	1.265744
141	48.82A.32	no	100 zeb	0	1.247076	0.268567
142	48.82A.32	yes	200 zeb	0	0	0.179044
143	P093XC	yes	200 zeb	0	5.793827	0.105686
144	48.82A.32	no	200 aza	0.00434745	0.895659	0.041031
146	48.82A.32	no	200 aza	0	0.561941	0.027149
147	P093XC	no	200 zeb	0.16589316	37.22648	3.84946
148	P093XC	yes	100 zeb	0.20242915	1.146232	0.178729
149	P093XC	yes	200 aza	0	6.292039	0.562204
150	48.82A.32	no	200 zeb	0.01283697	0.034057	0.033936
151	P093XC	yes	200 aza	0	0.540656	0.044117

152	P093XC	no	100 zeb	0.09380863	3.859089	2.475057
153	48.82A.32	yes	100 aza	0	0	0
154	P093XC	no	200 aza	0.04641059	11.85824	0.975091
155	48.82A.32	yes	none	0	0.569391	0.114251
156	48.82A.32	no	100 zeb	0.01579935	0	0.074659
157	P093XC	yes	none	0.00691221	5.071307	0.135744
158	P093XC	yes	100 zeb	0	4.40613	0.653831
159	48.82A.32	yes	200 aza	0.00987459	0	0.039592
160	P093XC	yes	100 aza	0	2.061516	0.22737
161	P093XC	no	200 zeb	0	11.34632	0.175335
162	P093XC	yes	200 zeb	0.00691221	0	0.111988
163	P093XC	no	200 aza	0.13034462	10.37676	0.60519
164	48.82A.32	no	100 zeb	0	1.082375	0.309948
165	P093XC	yes	200 aza	0	24.3593	0.064478
166	48.82A.32	yes	200 zeb	0.00197492	0	0.140268
167	48.82A.32	no	100 aza	0.02468648	0.006386	0.032805
168	P093XC	no	200 zeb	0	3.626011	7.401416
169	P093XC	no	none	0	0	0.022624
170	P093XC	no	none	0.02764886	5.113878	0.057691
171	48.82A.32	yes	none	0.0049373	0.848233	0.323522
172	P093XC	yes	200 aza	0	0	0
173	P093XC	yes	none	0.05036042	0	0.006787
174	P093XC	yes	none	0	0	0
175	48.82A.32	no	none	0.00888713	0.035121	0.059953
176	48.82A.32	yes	none	0	0	0
177	48.82A.32	no	none	0.00394984	0.020221	0.099545

B.2 Secondary Metabolite Data

B.2.1 Flavonoids:

Day 7

Table 8: *Concentrations of flavonoids for Day 7 samples. Concentration calculations were performed using a standard curve. Since caulcuations indicated the standards were not accurate, data was normalized to the mock elicited control flasks and presented in fold.*

Cell Line	5-Azacytidine /Zebularine	100/200 µM	Elicited	Concentration (µg/L)	Fold
48.82A.32	aza	200	yes	-0.0611456	0.893204
48.82A.32	-	-	yes	0.093176767	1.135518
48.82A.32	aza	100	no	0.015113867	1.012945
48.82A.32	zeb	200	yes	-0.006784967	0.97856
48.82A.32	zeb	100	yes	0.0107341	1.006068
48.82A.32	zeb	200	no	0.1622225	1.243932
48.82A.32	aza	100	yes	0.099875233	1.146036
48.82A.32	-	-	no	0.0068696	1
48.82A.32	zeb	100	no	0.113014533	1.166667
48.82A.32	aza	200	no	0.089312267	1.12945
48.82A.32	aza	100	yes	0.027995533	1.033172
48.82A.32	zeb	100	yes	-0.017347933	0.961974
P093XC	-	-	no	0.071535567	1
P093XC	zeb	200	no	0.101678667	1.042967
P093XC	zeb	200	yes	0.950666667	2.253153
P093XC	aza	100	no	0.177938133	1.151671
P093XC	zeb	100	yes	0.1184248.82A.3233	1.066838
P093XC	zeb	100	yes	0.042165367	0.958134
P093XC	aza	100	yes	0.079264567	1.011017
P093XC	zeb	100	no	0.115590867	1.062798
P093XC	-	-	yes	0.144961067	1.104664
P093XC	aza	200	no	0.1065737	1.049945
P093XC	aza	100	yes	0.087766467	1.023136
P093XC	aza	200	yes	0.097041267	1.036357

Day 14

Table 9: *Concentrations of flavonoids for Day 14 samples. Concentration calculations were performed using a standard curve. Since calculations indicated the standards were not accurate, data was normalized to the mock elicited control flasks and presented in fold.*

Cell Line	5-Azacytidine /Zebularine	100/200 µM	Elicited	Concentration (µg/L)	Fold
48.82A.32	aza	200	yes	0.121836233	0.993561
48.82A.32	zeb	200	yes	0.222919967	1.163258
48.82A.32	zeb	100	yes	0.190428767	1.108712
48.82A.32	aza	100	no	0.095662767	0.949621
48.82A.32	aza	100	yes	0.143497033	1.029924
48.82A.32	-	-	no	0.125672	1
48.82A.32	-	-	yes	0.0945346	0.947727
48.82A.32	zeb	100	yes	0.2048.82A.32693	1.132955
48.82A.32	zeb	100	no	0.138081833	1.020833
48.82A.32	aza	200	no	0.200131	1.125
48.82A.32	zeb	200	no	0.1398869	1.023864
48.82A.32	aza	100	yes	0.090247567	0.94053
P093XC	zeb	100	yes	0.113939067	0.855537
P093XC	zeb	200	no	0.1270258	0.874711
P093XC	zeb	100	no	0.132892267	0.883306
P093XC	aza	100	no	0.159968267	0.922975
P093XC	aza	100	yes	0.136728033	0.888926
P093XC	-	-	no	0.212540833	1
P093XC	zeb	200	yes	0.161322067	0.924959
P093XC	zeb	100	yes	0.1385331	0.89157
P093XC	-	-	yes	0.1581632	0.920331
P093XC	aza	100	yes	0.098370367	0.832727
P093XC	aza	200	yes	0.180049633	0.952397
P093XC	aza	200	no	0.140112533	0.893884
48.82A.32	zeb	100	yes	0.13998	0.935273
48.82A.32	zeb	100	no	0.116219333	0.892221

48.82A.32	zeb	100	no	0.158988533	0.969715
48.82A.32	aza	200	no	0.275006133	1.179929
48.82A.32	aza	200	yes	0.301060933	1.227138
48.82A.32	-	-	yes	0.211589733	1.065024
48.82A.32	zeb	200	yes	0.192908933	1.031176
48.82A.32	aza	100	no	0.251737067	1.137767
48.82A.32	zeb	200	yes	0.2191276	1.078682
48.82A.32	aza	100	yes	0.223715867	1.086995
48.82A.32	-	-	no	0.175702933	1
48.82A.32	zeb	200	no	0.216505733	1.073931
48.82A.32	aza	200	yes	0.195203067	1.035333
P093XC	zeb	100	yes	0.1552196	0.771775
P093XC	zeb	200	yes	0.1881568	0.81961
P093XC	zeb	200	no	0.3199056	1.010947
P093XC	aza	100	no	0.1095008	0.705378
P093XC	aza	100	yes	0.213556133	0.856497
P093XC	aza	100	no	0.299422267	0.981199
P093XC	-	-	no	0.312367733	1
P093XC	-	-	yes	0.171606267	0.795574
P093XC	aza	200	no	0.222732667	0.869824
P093XC	aza	200	no	0.2181444	0.86316
P093XC	zeb	200	yes	0.257308533	0.920038

Day 21

Table 10: *Concentrations of flavonoids for Day 21 samples. Concentration calculations were performed using a standard curve. Since calculations indicated the standards were not accurate, data was normalized to the mock elicited control flasks and presented in fold.*

Cell Line	5-Azacytidine /Zebularine	100/200 µM	Elicited	Concentration (µg/L)	Fold
48.82A.32	zeb	100	no	-0.023886667	1.247824
48.82A.32	zeb	200	yes	-0.034701867	1.209518

48.82A.32	aza	200	no	0.0529668	1.520023
48.82A.32	zeb	100	yes	-0.118437733	0.912943
48.82A.32	aza	100	yes	-0.100740133	0.975624
48.82A.32	zeb	100	yes	-0.124336933	0.892049
48.82A.32	-	-	no	-0.093857733	1
48.82A.32	zeb	200	no	-0.105492267	0.958793
48.82A.32	aza	100	no	-0.127778133	0.879861
48.82A.32	aza	200	yes	-0.102542667	0.96924
48.82A.32	zeb	200	yes	-0.06895	1.088218
48.82A.32	-	-	yes	-0.135479867	0.852583
P093XC	zeb	100	yes	-0.090908133	0.92019
P093XC	-	-	yes	-0.061576	1.014799
P093XC	aza	100	yes	-0.138429467	0.766913
P093XC	zeb	100	no	-0.0979544	0.897463
P093XC	aza	200	no	-0.115324267	0.841438
P093XC	zeb	100	yes	-0.061576	1.014799
P093XC	aza	100	no	-0.124173067	0.812896
P093XC	aza	200	yes	-0.152194267	0.722516
P093XC	aza	100	yes	-0.107130933	0.867865
P093XC	-	-	no	-0.066164267	1
P093XC	zeb	200	yes	-0.067147467	0.996829
P093XC	zeb	200	no	-0.090416533	0.921776

B.2.2 Phenolics:

Day 7

Table 11: *Concentrations of phenolics for Day 7 samples. Concentration calculations were performed using a standard curve. Since calculations indicated the standards might not be accurate, data was normalized to the mock elicited control flasks and presented in fold.*

Cell Line	5-Azacytidine /Zebularine	100/200 µM	Elicited	Concentration (µg/L)	Fold
48.82A.32	aza	200	yes	0.265975333	1.001313

48.82A.32	-	-	yes	0.267804667	1.007999
48.82A.32	aza	100	no	0.219752	0.832378
48.82A.32	zeb	200	yes	0.24974	0.941977
48.82A.32	zeb	100	yes	0.257155333	0.969078
48.82A.32	zeb	200	no	0.249968667	0.942813
48.82A.32	aza	100	yes	0.184994667	0.705349
48.82A.32	-	-	no	0.265616	1
48.82A.32	zeb	100	no	0.230858667	0.87297
48.82A.32	aza	200	no	0.253268	0.954871
48.82A.32	aza	100	yes	0.211748.82A.32667	0.803128
48.82A.32	zeb	100	yes	0.247257333	0.932904
P093XC	-	-	no	0.234386667	1
P093XC	zeb	200	no	0.24043	1.024933
P093XC	zeb	200	yes	0.256012	1.089218
P093XC	aza	100	no	0.2958	1.253369
P093XC	zeb	100	yes	0.252843333	1.076146
P093XC	zeb	100	yes	0.240038	1.023315
P093XC	aza	100	yes	0.264048.82A.32	1.122372
P093XC	zeb	100	no	0.254672667	1.083693
P093XC	-	-	yes	0.257188	1.09407
P093XC	aza	200	no	0.271920667	1.154852
P093XC	aza	100	yes	0.25268	1.075472
P093XC	aza	200	yes	0.279532	1.186253

Day 14

Table 12: *Concentrations of phenolics for Day 14 samples. Concentration calculations were performed using a standard curve. Since calculations indicated the standards might not be accurate, data was normalized to the mock elicited control flasks and presented in fold.*

Cell Line	5-Azacytidine /Zebularine	100/200 µM	Elicited	Concentration (µg/L)	Fold
48.82A.32	aza	200	yes	0.054099333	1.178549
48.82A.32	zeb	200	yes	0.040673333	0.923745
48.82A.32	zeb	100	yes	0.035479333	0.82517
48.82A.32	aza	100	no	0.005132	0.249225
48.82A.32	aza	100	yes	0.006928667	0.283323
48.82A.32	-	-	no	0.044691333	1
48.82A.32	-	-	yes	0.033715333	0.791692
48.82A.32	zeb	100	yes	0.013592667	0.409795
48.82A.32	zeb	100	no	0.067852	1.439554
48.82A.32	aza	200	no	0.022282	0.574706
48.82A.32	zeb	200	no	0.014964667	0.435834
48.82A.32	aza	100	yes	0.049036	1.082455
P093XC	zeb	100	yes	0.008562	0.956604
P093XC	zeb	200	no	0.0148.82A.3266667	1.320755
P093XC	zeb	100	no	0.008856	0.973585
P093XC	aza	100	no	0.028880667	2.130189
P093XC	aza	100	yes	0.079612	5.060377
P093XC	-	-	no	0.009313333	1
P093XC	zeb	200	yes	0.027443333	2.04717
P093XC	zeb	100	yes	0.099440667	6.20566
P093XC	-	-	yes	0.055504	3.667925
P093XC	aza	100	yes	0.014344	1.290566
P093XC	aza	200	yes	0.009346	1.001887
P093XC	aza	200	no	0.053576667	3.556604
48.82A.32	zeb	100	yes	0.221712	1.433055
48.82A.32	zeb	100	no	0.210442	1.362747
48.82A.32	zeb	100	no	0.1448.82A.3214667	0.953332

48.82A.32	aza	200	no	0.210082667	1.360505
48.82A.32	aza	200	yes	0.199466	1.294273
48.82A.32	-	-	yes	0.206424	1.337681
48.82A.32	zeb	200	yes	0.188	1.222743
48.82A.32	aza	100	no	0.202112	1.310781
48.82A.32	zeb	200	yes	0.228735333	1.47687
48.82A.32	aza	100	yes	0.127762667	0.846953
48.82A.32	-	-	no	0.152295333	1
48.82A.32	zeb	200	no	0.190972667	1.241288
48.82A.32	aza	200	yes	0.13949	0.920114
P093XC	zeb	100	yes	0.222561333	2.142033
P093XC	zeb	200	yes	0.185321333	1.796055
P093XC	zeb	200	no	0.187608	1.817299
P093XC	aza	100	no	0.122536	1.212747
P093XC	aza	100	yes	0.129494	1.27739
P093XC	aza	100	no	0.133348.82A.32667	1.313202
P093XC	-	-	no	0.099636667	1
P093XC	-	-	yes	0.233439333	2.243096
P093XC	aza	200	no	0.184047333	1.784219
P093XC	aza	200	no	0.19976	1.930197
P093XC	zeb	200	yes	0.124561333	1.231563

Day 21

Table 13: *Concentrations of phenolics for Day 21 samples. Concentration calculations were performed using a standard curve. Since calculations indicated the standards might not be accurate, data was normalized to the mock elicited control flasks and presented in fold.*

Cell Line	5-Azacytidine /Zebularine	100/200 µM	Elicited	Concentration (µg/L)	Fold
48.82A.32	zeb	100	no	0.166505333	

48.82A.32	zeb	200	yes	0.179833333	0.85213
48.82A.32	aza	200	no	0.188620667	0.917212
48.82A.32	zeb	100	yes	0.209200667	0.960121
48.82A.32	aza	100	yes	0.205607333	1.060616
48.82A.32	zeb	100	yes	0.190384667	1.043069
48.82A.32	-	-	no	0.196787333	0.968735
48.82A.32	zeb	200	no	0.210605333	1
48.82A.32	aza	100	no	0.119171333	1.067475
48.82A.32	aza	200	yes	0.054164667	0.620992
48.82A.32	zeb	200	yes	0.183949333	0.303557
48.82A.32	-	-	yes	0.210768667	0.937311
P093XC	zeb	100	yes	0.193128667	1.068272
P093XC	-	-	yes	0.182316	0.976681
P093XC	aza	100	yes	0.191299333	0.924175
P093XC	zeb	100	no	0.20515	0.967798
P093XC	aza	200	no	0.210572667	1.035057
P093XC	zeb	100	yes	0.206097333	1.06139
P093XC	aza	100	no	0.186432	1.039657
P093XC	aza	200	yes	0.130964	0.944162
P093XC	aza	100	yes	0.167746667	0.67481
P093XC	-	-	no	0.197930667	0.853426
P093XC	zeb	200	yes	0.173496	1
P093XC	zeb	200	no	0.147885333	0.881345