Increasing Production of Artemisinin by Demethylation of

***A. annua* Hairy Root Cultures.**

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Joslyn A. Foley

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Professor Pamela J. Weathers, Advisor

ABSTRACT

This study explored the role of hypermethylation of DNA in transformed hairy roots of *Artemisia annua* in diminishing production of artemisinin when maintained in culture for long periods. Two weeks following treatment with 5-azacytidine roots showed inhibited growth, but no change in artemisinin production. HPLC was used to analyze artemisinin content. DNA in treated showed reduced methylation compared to controls when compared through TLC and quantitated using spectroscopy. Analysis of the mitotic figures in the root tips indicated that 5-azacytidine inhibits growth by arresting cell division in metaphase, preventing elongation of the roots.

ACKNOWLEDGEMENTS

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TABLE OF CONTENTS

TABLE OF FIGURES

TABLE OF TABLES

INTRODUCTION

1.1 SIGNIFICANCE OF ARTEMISININ

Malaria is a disease that causes over 1 million deaths each year, putting 40% of the world population at risk of infection (WHO, 2004). There are four parasites that cause malaria, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, and *Plasmodium falciparum*. Of these four, only *Plasmodium falciparum* causes severe, potentially fatal malaria (CDC, 2004). Recently some strains of *Plasmodium falciparum* have shown resistance to many of the current affordable treatments include chloroquine, quinine, mefloquine, and primaquine (Balint, 2001), making the need for new effective treatments even more urgent.

An antimalarial drug that has been used as a folk remedy since 168 B.C. in China, was named qinghaosu, but is now better know as artemisinin, a sesquiterpene from the plant *Artemisia annua L.* (Meshnick *et al*, 1996). Artemisinin was first purified and its molecular structure determined in 1972. Artemisinin and its derivatives have been found to be effective against all stages of resistant strains of *P. falciparum* (Balint, 2001).

Although artemisinin has been found to be a useful medicine, its production is very low in comparison with what is actually needed to treat the worldwide threat of malaria. The World Health Organization, WHO, estimated that at least 130 million treatments would be needed in 2006, requiring 330 tons of artemisinin (WHO, 2004). This presents a problem because of the very low production levels of artemisinin in the native plant. One ton of dry *A. annua* leaves produce only 6 kg of artemisinin (Van Geldre *et al.,* 1997). The complex biosynthetic pathway for artemisinin has prevented it

from being produced by organic synthesis (Abdin *et al*., 2003) and research has mainly focused on increasing artemisinin production in *A. annua* plants. However, recent work has also included engineering pathways in both yeast and *E. coli* to produce precursors to artemisinin (Ro *et al*., 2006 and Martin *et al*., 2003).

1.2 Chemistry and Source of Artemisinin

Artemisinin is an endoperoxide sesquinterpene lactone in the terpenoid family of secondary metabolites (Figure 1). Its molecular formula is $C_{15}H_{22}O_5$. The key structural part of its antimalarial function is its endoperoxide bridge. It has been proposed that the endoperoxide bridge is cleaved by a heme group to form a free radical that causes selective alkylation of parasite proteins, leading to parasite death (Pandey *et al*.1999). Pandey *et al*. (1999) also proposed that artemisinin forms a complex with heme that possibly interrupts the parasite's hemoglobin catabolism.

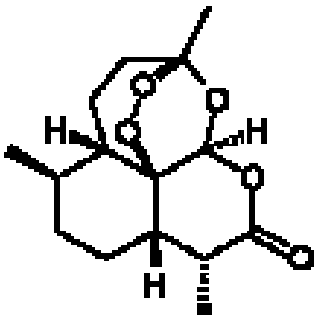

Figure 1. The chemical structure of artemisinin.

The attempts that have been made to synthetically produce artemisinin have proven to be both inefficient and costly (Abdin *et al*., 2003). Although these attempts were impractical at a large enough scale to be useful, it should be noted that artemisinic acid was a major intermediate. This is interesting because *A. annua* produces eight to ten times more artemisinic acid than artemisinin in some chemotypes (Abdin *et al*., 2003). A method for converting artemisinic acid to artemisinin would be invaluable for increasing production efficiency.

In *A. annua* artemisinin is found mainly in the shoots of the plant, however, in this study we will be focusing specifically on hairy root cultures which produce less artemisinin than the shoots, but more than in normal roots. Hairy roots are actually a type of tumorous growth in plants caused by infection of the bacterium, *Agrobacterium rhizogenes* (Hu and Du, 2006). The hairy roots are a byproduct of the infection and usually produce extensive amounts of secondary metabolites. Unlike normal plant tissue cultures, secondary metabolite production by hairy roots is relatively stable and at a level equivalent to or greater than the parent plant (Giri and Narasu, 2000). Indeed, Weathers *et al*. (1994) showed that transformed roots of *A. annua* also produce artemisinin.

1.3 Production of Artemisinin

1.3.1 Biochemical Pathway

Artemisinin is a sequiterpenoid synthesized from five units of isopentyl diphosphate (IPP). IPP is produced in the cytoplasm, mitochondria, and plastid. The

pathways for the synthesis of terpenoids include the mevalonate pathways located in the cytosol, and the non-mevalonate pathway localized to the plastid (Figure 2) (Croteau *et al.* 2000).

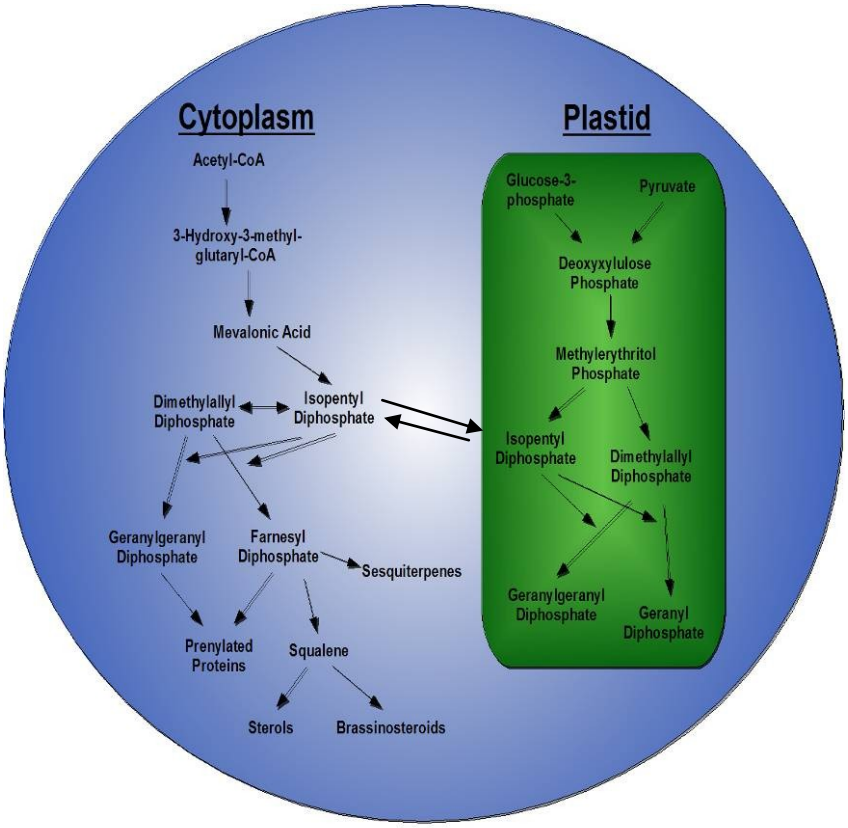

Figure 2. Terpenoid biosynthesis pathways.

In the chloroplast IPP is derived from pyruvate and in the cytosol IPP is derived from acetyl CoA. IPP is a precursor to a number of compounds, including farnesyl diphosphate (FPP), which is then converted into sesquiterpenes, including artemisinin. FPP is also a branch point for several other terpenoid pathways, including sterols, prenylated proteins and brassinosteroids (Figure 2) (Croteau *et al.,* 2000). There have been several studies on the manipulation of these pathways in attempts to shift production

away from some of these other products with the hope that more FPP will be directed toward sesquiterpene production (Rodríguez-Concepción *et al*., 2004). For example, the sterol inhibitor, miconazole, inhibits squalene synthase, which is the first regulatory step leading to sterols which in turn coordinately up regulates production of sesquiterpenes (Abdin et al., 2003). However, there is much about the regulation of these pathways that is still unknown and being studied.

1.4 Increasing Production of Artemisinin

Although it has proven effective in curing malaria, there are several factors that have prevented the commercial production and widespread use of artemisinin. Many factors can affect production of artemisinin and many attempts have been made to improve production yields. Research has focused mainly on increasing the production of artemisinin in *A. annua* as the yield of artemisinin is only in the range of 0.01 - 0.8 % of plant biomass with the highest yield in any particular clone reaching only 1.1% (Abdin *et al*., 2003). Several approaches have been taken to optimize artemisinin yield including selective breeding, hormone treatment, infection with *Agrobacterium rhizogenes*, and genetic engineering to alter the expression of genes associated with the biosynthetic pathway of artemisinin (Delabays, 1993, Weathers *et al*., 1997, 1994, and Abdin *et al.,* 2003). Recent work has also been done on engineering a mevalonate pathway to synthesize terpenoids in *E. coli* and yeast (Martin *et al*., 2003 and Ro *et al.*, 2006).

1.4.1 Transformed Hairy Roots Lose Productivity Over Time

Further complicating the commercial production of artemisinin, it has been shown that transformed hairy roots of *A. annua* that are maintained in culture for greater than 10 years may lose their ability to produce artemisinin (Kim, 2001). This loss of productivity is not uncommon for *in vitro* cultures, many of which have been shown to slowly lose phenotypic characteristics as they are maintained in culture for many generations (Street *et al*., 1977). Determining the cause of this loss of productivity, although challenging, is crucial for understanding and stabilizing artemisinin synthesis.

One possible reason for loss of artemisinin production is that the genes for artemisinin production are slowly being silenced by the accumulation of methylated DNA. Paszkowski and Whitham (2001) have reported that methylation is responsible for some gene silencing in a variety of plant cultures. For instance, this was found to be the case with sorghum, a significant commercial crop. Genetic engineering to improve sorghum cultivars was most successful when the plant was transformed with *Agrobacterium rhizogenes*. In sorghum, transgene silencing resulted in reduced transformation efficiency while treatment with the demethylating agent, 5-azacytidine, resulted in reactivation of the silenced gene (Emani *et al*., 2002).

1.4.2 DNA Methylation in Plants

DNA methylation is the addition of a methyl group, for example, to the number 5 carbon of cytosine to form 5-methylcytosine (Figure 3). Usually this occurs in cytosines

that are part of CpG, CpNpG, or CpNpNp sequences, where p denotes the phosphate backbone of the DNA and N is any nucleotide.

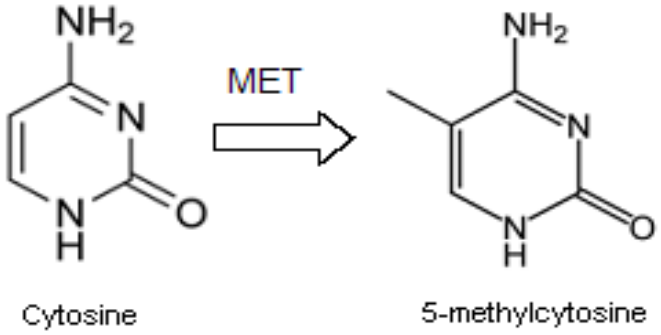

Figure 3. A cytosine residue is modified by the addition of a methyl group at the number 5 carbon to form 5-methylcytosine. Met, methyltransferase, catalyzes this reaction.

Methyl groups are transferred from S-adenosyl-methionine to the cytosine residues by methyltransferases in newly replicated DNA (Bird, 1978). The genes for 2 types of methyltransferases have been identified in *Arabidopsis*. The first class includes METI and METII, a third proposed methyltransferase, METIII, belongs to the second class (Finnegan *et al*, 1998). METIII has a chromodomain, a short motif that is found in chromatin associated proteins. Methyltransferases preferentially bind to CpG, CpNpG, and CpNpNp sites in repeat sequences which offer a possible mechanism for allowing the methylation pattern to be conserved during replication. Unlike animals, plant DNA does not undergo universal demethylation during embryo development (Finnegan *et al*., 1996, Kakutani *et al*., 1995). This is demonstrated in *Arabidopsis* plants with mutations that result in reduced methylation that subsequently generate progeny that also show reduced

methylation even if they do not have the mutation (Finnegan *et al*., 1996). Methylation has been shown to also change during gametogenesis, as young tomato and *Arabidopsis* seedlings show methylation levels that are about 20% lower than in mature plants (Messeguer *et al*., 1991; Finnegan *et al*., 1998). It is thus reasonable to hypothesize that DNA methylation may result in the accumulation of methylation errors in hairy roots leading to decreased artemisinin production.

1.4.3 Effects of Methylation of Gene Function

Methylation is necessary for normal plant development. Altered methylation results in a variety of abnormalities in most species of plants. Reduced methylation in *Arabidopsis,* for example, results in loss of apical dominance, reduced stature, altered leaf size and shape, reduced root length, homeotic transformation of floral organs and reduced fertility (Finnegan *et al*., 1996; Kakutani *et al*., 1995; Ronemus *et al*., 1996). It has even been suggested that in animal males, female sex suppression is the result of methylation of a specific sequence on the Y chromosome or autosomes (Janousek *et al*., 1996).

Methylation of plant DNA has been shown to have two roles, regulation of gene expression and protection of the genome. DNA methylation affects transcription by interfering with transcription factor binding and altering chromatin formation resulting in a repression of transcription of the methylated regions (Kass *et al*., 1997). Mobile elements of the genome as well as repeated sequences are methylated in plants, possibly as a way to recognize and then silence foreign DNA. (Finnegan *et al*., 1998).

1.4.4 DEMETHYLATION

Methylation patterns change during normal plant development, so demethylation of plant DNA is probably a natural occurrence. Although the usual trend in plants is to increase methylation as the plant ages, there is evidence that plants have the ability to demethylate certain parts of the genome. The best example of this is that plants with vernalization-dependent flowering, flower early when treated with the demethylating agent, 5-azacytidine, indicating that in these plants vernalization is mediated by demethylation in the promoter regions of genes necessary for the initiation of flowering (Burn *et al*., 1993).

A common demethylating agent, 5-azacytidine (Figure 4) was used in this study. It functions by displacing cytidine residues in the DNA (Doerfler, 1983) and in mice has been shown to covalently bind to methyltransferases causing them to become inactivated (Jutterman *et al*., 1994).

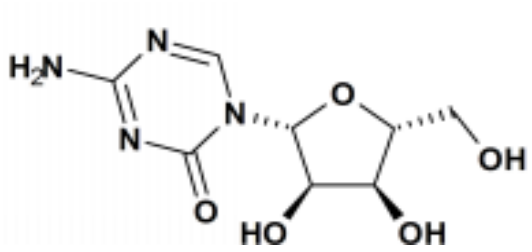

Figure 4. The chemical structure of 5-azacytidine, a cytidine analog that results in demethylation.

In sorghum 5-azacytidine was shown to be successful in the reactivation of a transgene that was silenced due to methylation (Emani, *et al*., 2002). However, because 5-azacytidine is a nonspecific demethylating agent it also has been shown to cause a number of undesirable morphological characteristics similar to those found in plants with mutations resulting in reduced methylation. For example, methylation inhibitor studies with rice, flax, tobacco and *Triticale* showed similar but variable effects including dwarfing and reduced fertility (Richards, 1997).

In a previous study by Woerdenbag *et al*. (1993) the use of 5-azacytidine to increase artemisinin production in *A. annua* shoots was unsuccessful. In that study initial 5-azacytidine concentrations of 4 and 8 μM were gradually increased to 12 and 24 μM, respectively. These concentrations were concluded to be toxic to the cultures (Woerdenbag *et al*., 1993). However, later work treating transformed hairy root cultures of *A. annua* with 5-azacytidine at concentrations of 10, 20, and 50 μM inhibited root growth, but were not toxic; when 5-azacytidine was removed from the cultures, the roots grew normally (Fuller, 2004). Treatment with 5-azacytidine significantly reduced root growth, and furthermore appeared to decrease artemisinin production by greater than 50% even in 10 μM concentrations of 5-azacytidine, although these results were not statistically significant (Fuller, 2004).

Although the initial results of the Fuller (2004) study were not promising, he also found that after the removal of 5-azacytidine and subsequent inoculation into B5 medium the hairy root cultures of *A. annua* grown in 20 and 50 μM grew at rates of 0.41 and 0.43g FW/day which is similar to the normal growth rate of the YUT16 clone, 0.45g

FW/day, as reported by Towler and Weathers (2003). On the other hand, roots grown in 10 μM 5-azacytidine showed accelerated growth, 1.4 times the normal rate, at 0.64g FW/day; possibly indicating that at that concentration 5-azacytidine had inhibited root cell elongation, but had not affected cell division (Fuller, 2004). Unfortunately, these rapidly growing cultures were not assayed for artemisinin. These observations suggested that growing hairy roots on normal medium following treatment with 5-azacytidine may also affect the artemisinin yield.

Interestingly, a comparison of the methylation levels of *A. Annua* shoots, roots, and transformed roots showed that the shoots are the least methylated portion of the plant, and the roots are the most heavily methylated. Transformed roots of *A. annua* showed slightly less methylation than normal roots. This pattern corresponds to the established levels of artemisinin production in the plant, as the shoots produce a large amount of artemisinin, while the roots produce almost none and transformed roots have been shown to produce amounts between these two (Ferreira and Janick, 1996). Further experimentation was deemed necessary to determine the relationship between methylation and artemisinin production. If transformed roots grown in medium containing 5-azacytidine then grown out on normal medium show decreased methylation and increased artemisinin production it would be reasonable to conclude that methylation probably plays a role in artemisinin production. Furthermore, if transformed root cultures showing decreased artemisinin production were also found to have higher levels of methylation over time corresponding to this decrease it would provide evidence that methylation also plays a role in the habituation of these cultures over time.

2. Hypothesis and Objectives

2.1 Hypothesis

Based on the earlier study by Fuller, it is proposed that addition of 5-azacytidine to *Artemisia annua* first will result in increased growth and artemisinin production, but only after subsequent grow out of the roots in 5-azacytidine media.

2.2 Objectives

This project has two main objectives:

- To determine if treatment of *A. annua* hairy root cultures with 5-azacytidine results in demethylation of their DNA by comparing the level of methylation in treated roots with untreated roots and correlating this with the production of artemisinin after subsequent growth on 5-azacytidine-free medium.
- To determine the level of mitosis in roots treated with 5-azacytidine and untreated controls by measuring the number of mitotic figures in root tips.

3. METHODS

3.1 CULTURE CONDITIONS

Clones of *Artemisia annua* L. (clone YUT16) (Weathers *et al*. 1994) hairy roots were used in the experiments. The cultures were maintained in 125mL Erlenmeyer flasks containing 50 mL autoclaved Gamborg's B5 basal medium (Gamborg *et al.,* 1968) pH 5.7 with 3% (w/v) sucrose. They were kept at 25°C under continuous fluorescent white light on an orbital shaker at 100 rpm. Flasks were subcultured every 14 days.

Fourteen-day-old cultures were aseptically blotted dry on a pre-sterilized maxi-pad and 0.4g of fresh weight was weighed in a sterile Petri dish, and put into a 125mL flask with 50mL of autoclaved Gamborg's B5 basal medium with 3% (w/v) sucrose, at a pH of 5.7. A solution of filter sterilized (0.22µm) 5-azacytidine (12.21mg in 5mL dH_2O) was prepared. To six experimental flasks of roots, 0.10mL of the 5-azacytidine solution was added to make a 20µM solution. Control cultures contained no 5-azacytidine. The cultures were kept at 25ºC under continuous fluorescent white light on orbital shaker at 100 rpm. After 14 days all the cultures were provided fresh medium. If there was a lot of growth, e.g. the controls, then the biomass was also reduced to the level of the initial inoculum. If no growth occurred, the medium was just replaced. After 28 days the cultures were rinsed with fresh medium to remove any remaining 5-azacytidine, subcultured again and all were fed media without 5-azacytidine. After an additional 14

days (42 days total) the roots were harvested, weighed, and extracted with toluene for analysis of artemisinin and level of DNA methylation.

3.2. Extraction and Analysis of Artemisinin

Roots were rinsed with diH2O, blotted dry and fresh weight obtained. Each sample (2 g FW if available) was placed in a test tube with 1mL of toluene per gram of root and extracted by sonication in an ice bath for 30 minutes. Samples were centrifuged for 1-2 minutes at about 5000xg and the supernatant was removed and placed in new test tubes. Tubes were re-extracted twice using this same procedure and each sample's pooled extracts were dried under nitrogen at 30°C. Samples were stored at -20ºC until HPLC analysis.

HPLC analysis of the Q260 derivatized artemisinin was performed according to Smith *et al*. (1997). Samples were re-suspended in 100µL of methanol. A 400µL aliquot of 0.2% (v/w) NaOH was added and the tubes were capped and heated at 50°C for 35 minutes. Then the tubes were placed in ice water and 400µL of 0.2 M acetic acid was added followed by another 100µL of methanol. Samples were vortexed and then syringe filtered though a 0.22µm membrane (FP-200 Vericel) directly into a HPLC sample vial.

HPLC parameters:

- UV detector set at 260 nm

- 15 cm Microsorb-MV C18 column, 4.6 mm i.d., 5µm beads with 100A pore size
- mobile phase of 55% 0.22µm filtered 0.01M phosphate buffer pH 7, 45% methanol, final pH adjusted to 7.0
- 1.0mL/min mobile phase flow rate
- A 10µg/mL artemisinin standard was injected for quantitative comparison.

3.3 Methylation Studies

3.3.1 DNA Extraction

To determine the overall methylation levels of both treated and untreated roots DNA was extracted using a modification of the method described by Schuler (1989). After two weeks growth on regular Gamborg's B5 medium without 5-azacytidine, 10g of roots from both the control and the experimental group treated with 5-azacytidine were analyzed. The root tissue was wrapped in aluminum foil, dipped in liquid nitrogen and crushed using a mortar and pestle to yield a fine powder. This powder was placed in a graduated cylinder with 10 volumes of grinding buffer. The grinding buffer was prepared according to Lilly *et al.* (2001): 0.45M sorbitol, 50mM Tris buffer, pH 7.6 (prepared as described by Romangnano (2003)), 5mM EDTA, 0.2% BSA, 1.0% polyvinylprrolidone-360, 0.025% spermidine, 0.025% spermine, and 1mL of ß-mercaptoethanol. Diethyldithiocarbonate was then added to a concentration of 0.1M and the solution was iced for 10 minutes, transferred to a blender and pulsed on the highest setting for 5

seconds, allowed to settle, and pulsed again at the same speed for another 5 minutes. The homogenate was then washed through two layers of cheese cloth with grinding buffer into 50mL conical tubes then transferred to centrifuge tubes.

The resulting homogenate was pelleted at 350 x g for 10 minutes, and the supernatant was discarded. The pellet was then resuspended by gently shaking in 5 mL ice cold lysis buffer. The lysis buffer was prepared as described by Ross et al. (1999) using 10mM NaCl, 1 mM EDTA, 10% glycerol, and 10 mM β-mercaptoethanol, in 50 mM of Tris-HCl buffer, pH 8.0. DNA was then extracted by adding 2.5 mL of chloroform and 2 .5 mL of phenol, gently shaking for 30 min and allowing to settle. The top (aqueous) layer was removed using a transfer pipette and the volume measured. Next, 2 volumes of TE buffer (10 mM Tris-Cl, pH 7.5; 1 mM EDTA (Sosnick Group University of Chicago, 2005)), 2 volumes ethanol, and 0.1 volume of 2 M NaCl were added, the solution was gently mixed and then chilled at −80 °C for 30 min to precipitate the DNA.

The solution was then centrifuged at 4,000 x g for 10 min and the supernatant was discarded. The resulting pellet was resuspended in 900 µL dH_2O, mixed gently, and then reprecipitated by adding 100µL 2M NaCl and 2 mL ethanol and chilling at − 80°C for a minimum of 15 min. The solution was then centrifuged again at 43,000 x g for 10 min and the supernatant discarded.

The purity of the DNA sample was ensured by measuring the OD_{280} of the supernatant and comparing against a blank of 100 µL 2M NaCl, 900 µL water, and 2 mL ethanol. If an absorbance was seen the resuspension process was repeated. To quantify

the amount of DNA present in the sample, the concentration and the OD_{260} of a water blank and solutions containing 0 .1mg/mL and 0 .02mg/mL DNA were plotted. A best-fit line was found and the equation of this line, y = 0.082051x + b, with y being the concentration, b=0, and x being the OD_{260}, was used to relate the concentration of DNA in each sample to their OD_{260}. The purified pellet was dissolved in 2 mL water and the concentration (mg/mL) of DNA in the sample was quantified using this equation. All DNA samples were held at −20 °C between testing periods.

3.3.2 TLC Methylation Analysis

A TLC method was used to analyze the methylation levels of the DNA extracted from the roots grown in medium containing 5-azacytidine as compared to the control group (Fuller, 2004). The separation of the DNA bases was detectable with UV light to a sensitivity of 5 µg for each base (Table 1).

Table 1. TLC parameters and TLC sensitivity under short and long wave ultraviolet light (fuller, 2004).

Base	**TLC Sensitivity**	**RF (x100)**	**Visibility**		**Preferred Solvent**
			SUV	LUV	
Adenine	5 µg	67	x		Methanol
Thymine	5 µg	76	x		Hot Methanol
Guanine	5 µg	18		x	Weak Acid & Hot Ethanol
Cytosine	5 µg	24/47	x	x	Hot Methanol
5-methylcytosine	5 µg	36/47	x	x	Methanol

SUV = Short wave UV 260nm; LUV = long wave UV 280nm.

Known quantities of DNA in 1 mL were hydrolyzed in 80% formic acid at 145°C for 45 minutes and dried under nitrogen. These samples were later resuspended in a solution of 80µL H_2O and 20µL formic acid before being loaded onto a glass backed silica gel 60 TLC plate. For identification of the methylated bases 0.05 mg of the standards cytosine and 5-methylcytosine were also spotted. TLC analysis was performed using a chloroform:methanol:ammonium hydroxide (90:30:1) mobile phase. Long and short wave UV was used to analyze the plates according to their known visibilities (Table 1).

The resulting bands corresponding to 5-methylcytosine were identified, and resuspended in 1.2 mL of H_2O after being individually scraped from the plate, vortexed, sonicated, and finally microfuged for 3 min. The concentration of 5-methylcytosine in each supernatant was determined in the same way as the DNA concentration, by comparing the OD_{260} against an H_2O blank and the supernatant eluted from scraping the 5-methylcytosine standard. The percent of DNA methylation in each sample was obtained by dividing this concentration by the original concentration of the DNA sample.

3.3.3 Data Analysis

Eight replicates were used in all experiments and matched against at least three controls. Non-parametric tests were used to analyze all root growth and artemisinin production data due to the small sample sizes. The Mann-Whitney Wilcoxon (Petruccelli

et al., 1999a) and Friedman (Petruccelli *et al*., 1999b) tests were used to calculate statistical significances. TLC RF's were calculated based on the center of observed spots and relative to the distance the solvent front traveled from the origin.

3.4 Analysis of Chromosome Formation

To stain mitotic figures in root cells, samples of root tips from all cultures were transferred to cold Carnoy's solution (3:1, 100% ethanol: glacial acetic acid) and fixed overnight. They were rinsed twice diH_2O, blotted to remove excess water, and stained in aceto orcein solution and heated on a hot plate until the solution began to fume. Stained root tip meristems were removed and rinsed with Carnoy's solution then placed on a clean slide, and squashed. Cells were observed using light microscopy and at least ten fields of root tips cells per experimental treatment were counted. At least 100 cells per condition were counted.

4. Results and Discussion

4.1 Growth and Artemisinin Content

Addition of 5-azacytidine to *A. annua* hairy root cultures resulted in decreased growth (Figure 5), which contrary to the study done by Fuller (2004), did not return to

near the normal rate of 0.45g FW/day (Towler and Weathers, 2003) when switched into normal B5 medium (Figure 6).

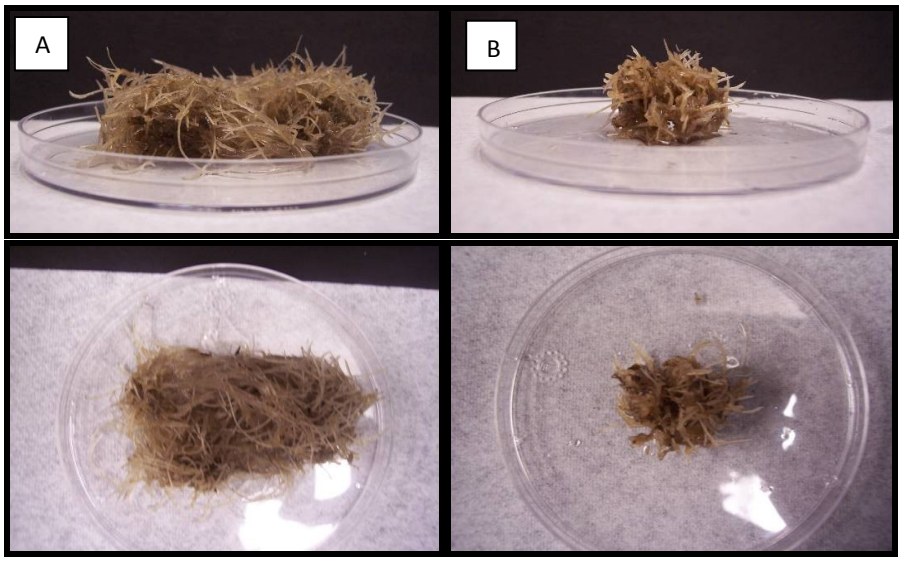

Figure 5. *A. annua* hairy root cultures grown in normal B5 medium (A); and cultures two weeks after being grown in medium containing 5-azacytidine (B). Roots in bottom row are the same as each culture in the row above, but viewed form a different perspective.

Fuller (2004) only observed accelerated growth after the removal of 5-azacytidine in cultures containing 10µM 5-azacytidine, half the concentration used in this experiment. The cultures grown in 20µM 5-azacytidine were observed to grow at a rate of 0.41g FW/day, similar to the normal growth rate. However, in this study the average growth rate of cultures removed from 20µM 5-azacytidine for 14 days was 0.075g FW/day.

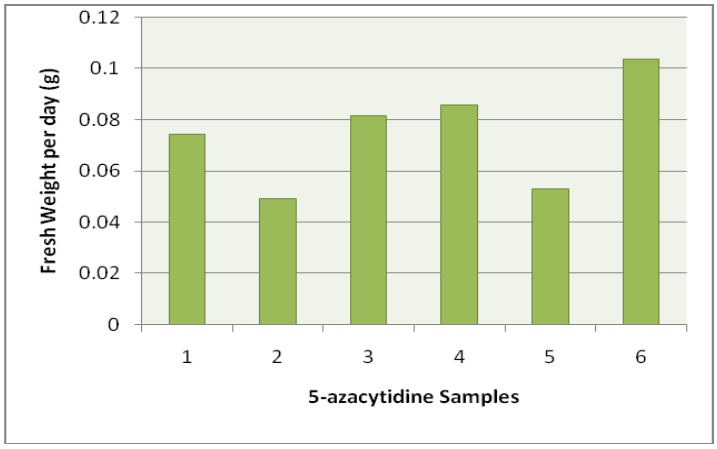

Figure 6. Root growth is slowed after treatment with 5-azacytidine, even after removal from culture medium.

Although root growth was inhibited, the concentration of artemisinin found in roots two weeks after treatment with 5-azacytidine was approximately the same as in control roots (Figure 7). These data are not statistically significant however, as 5-azacytidine roots needed to be pooled to obtain enough plant mass for HPLC analysis of artemisinin. There was no evidence of increased artemisinin concentrations in roots two weeks after removal from 5-azacytidine medium.

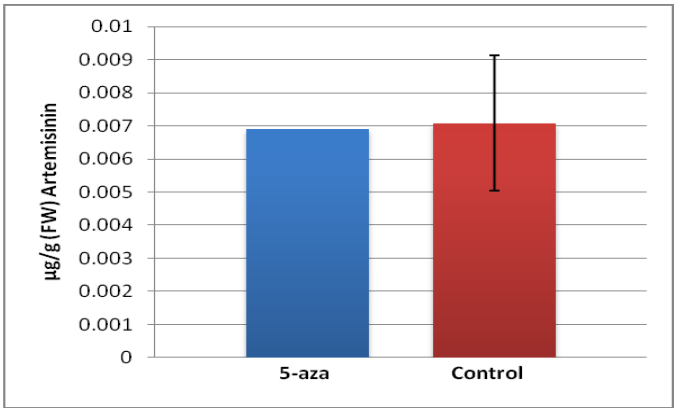

Figure 7. The 5-azacytidine roots and controls had similar concentrations of artemisinin.

4.2 TLC Methylation Analysis

TLC of formic acid hydrolyzed DNA effectively separated the four primary bases, adenine, thymine, guanine, and cytosine. Although previous work showed that formic acid is also effective in separating cytosine from 5-methylcytosine, it is possible that there is some demethylation of methylated cytosines during hydrolysis, as this has been shown to be the case with stronger acids. The amount of demethylation if any, due to chemical hydrolysis should be determined.

The TLC showed only a spot corresponding to thymine for the control roots; it is possible that the concentration of DNA was too low to visualize the other spots (Figure 8).

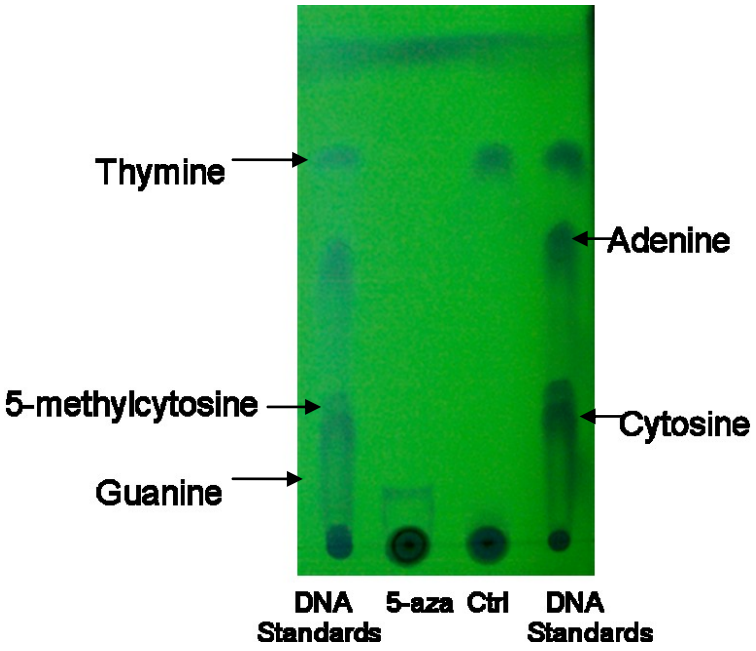

Figure 8. TLC comparison of hydrolyzed bases from 5-aza and control roots with DNA standards using a chloroform:methanol:ammonium hydroxide (90:30:1) mobile phase.

Nevertheless, the relative percent methylation for both the 5-azacytidine roots and controls was approximately determined from scrapings of the spots on the plate in the horizontal positions corresponding to 5-methylcytosine as determined from the DNA standards. The data suggested that the relative percent of 5-methylcytosine in the 5-azacytidine roots was lower than the control roots (Figure 9).

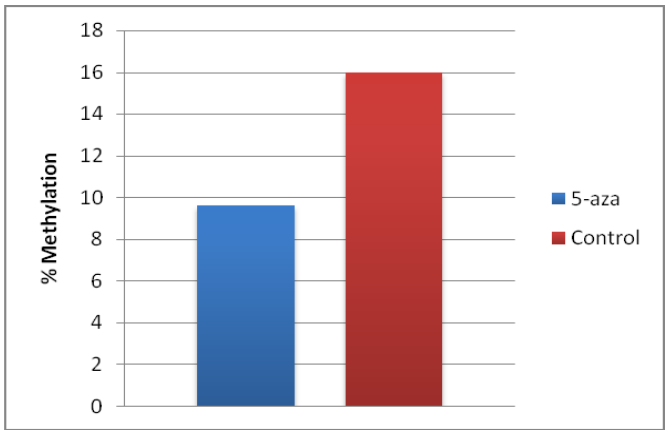

Figure 9. Percent 5-methylcytosine in hydrolyzed 5-aza and control roots.

4.3 Chromosome Formation

To determine if 5-azacytidine roots contained more cells undergoing mitosis than the cells in untreated roots, the cells in both 5-azacytidine and control root tip meristems were counted along with the number of cells whose chromosomes are visibly in some stage of mitosis. Several stages of mitosis were visible (Figure 10).

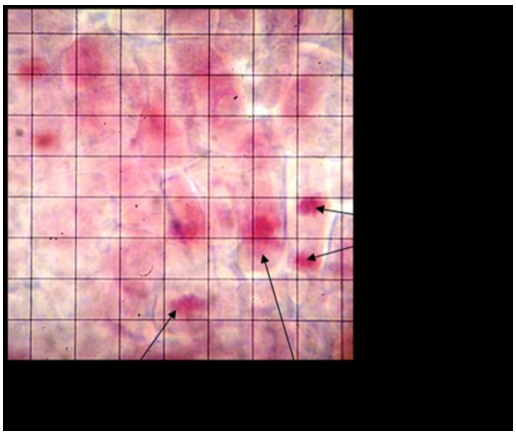

Figure 10. Cells of a 5-azacytidine treated root after staining with aceto orcein.

The percentage of the total number of cells counted for each the 5-aza and the control roots is summarized in Table 2. The percent of cells undergoing some phase of mitosis was significantly higher than in control cells ($p=0.01$). These results are consistent with my hypothesis that in 5-azacytidine the cells are undergoing active mitosis, but are arrested in their ability to elongate.

Table 2. Percent of total cells counted undergoing mitosis for 5-aza roots and control roots.

	# of Cells	% Mitosis
5-aza	123	14.6%
Control	194	2.6%

5. CONCLUSIONS

Although after two weeks in normal B5 medium roots grown in 5-azacytidine did not show rapid growth as seen in the study done by Fuller (2004) a 20μM concentration of 5-azacytidine did not prove toxic to *A. annua* hairy root cultures. The roots did show some regeneration after removal of 5-azacytidine and a visualization of the cells in the root tips showed that cells were still dividing, and at a rate significantly higher than normal roots. This supports the idea that 5-azacytidine inhibits growth of transformed roots by preventing cell elongation, but does not hinder cell division.

Treatment with 5-azacytidine was not shown to have increased artemisinin production, but unlike what was observed by Fuller (2004) they did not show that the concentration of artemisinin had decreased from that found in control roots. This may indicate that the roots recover their ability to produce artemisinin after treatment with 5-azacytidine.

The concentration of 5-methylcytosine from the genomic DNA of 5-azacytidine roots was less than control roots. Although this data is not statistically significant, it does support the hypothesis that 5-azacytidine effects the root cultures by decreasing the overall methylation of the DNA.

Future work should contain more replicates of 5-aza treated roots to allow a more detailed and statistically significant analysis of the effects of demethylation on artemisinin production. A lower concentration of 5-azacytidine should also be used, as Fuller (2004) showed that 10μM concentrations showed the best recovery and increased

growth after removal of 5-azacytidine from the medium. It should also be determined if hydrolysis of DNA into individual bases results in some demethylation of 5-methylcytosine. Finally, the 5-azacytidine roots should be allowed to undergo 2-3 subculturing cycles after 5-azacytidine is removed to determine if they are able to return to a normal growth rate and if artemisinin production increases after roots have recovered.

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