

**The effects of 5-azacytidine on transformed hairy root
cultures of *Artemisia annua***

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Abstract

This MQP compared the effects that varying levels of 5-azacytidine had on growth and artemisinin levels in transformed hairy roots of *Artemisia annua*. Also compared was the difference in DNA methylation levels between shoots, roots and transformed roots. The intent of this project was to test the effects DNA methylation may play in slowing artemisinin production in older hairy root cultures of *Artemisia annua* by attempting to demethylate the DNA of older root cultures through the incorporation of 5-azacytidine. Although the results showed that 5-azacytidine is an inhibitor of growth in hairy roots of *Artemisia annua*, there was no apparent increase in artemisinin.

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1 Introduction

1.1 Significance of artemisinin

One million people die and over 100 million more are infected with malaria each year (Abdin et al., 2003). Currently, most cases of malaria are treated with a relatively inexpensive drug called chloroquine. Chloroquine and other current treatments are based on the first effective antimalarial drug, quinine. Recent studies show, however, that the parasite responsible for the majority of fatal infections, *Plasmodium falciparum*, is growing increasingly resistant to quinoline-based drugs (Abdin et al., 2003; Board on Global Health, 2004). An alternative antimalarial for quinoline-based drugs, artemisinin, is currently the only replacement that is not showing a similar buildup of resistance. The current cost of treatment with artemisinin is a few dollars a course (Board on Global Health, 2004). However, this is relatively expensive for the world's poor and it is estimated that costs need to be reduced to around \$0.10 to be affordable to the majority of those infected; as a result of the high cost of this drug, deaths from malaria are rising (Board on Global Health, 2004).

Artemisinin is a rather complex sesquiterpene lactone produced in the plant *Artemisia annua* L. (Fig. 1). Its unique endoperoxide bridge is responsible for its antimalarial activity. Specifically, artemisinin inhibits a calcium ATPase called PfATP6, the SERCA orthologue of *Plasmodium falciparum*, causing the death of the *Plasmodium falciparum* parasite within

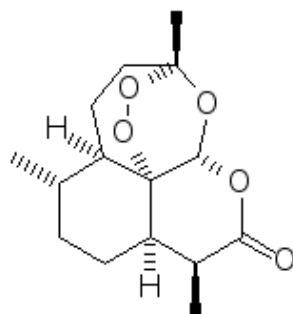


Figure 1: Chemical structure of artemisinin

the host's erythrocytes (Board on Global Health, 2004).

The endoperoxide (O-O) bridge is very unique to this species and is chemically complex to mimic (Gelder et al., 1997). Large scale chemical manufacture of the drug would be extremely involved and costly and thus, commercially unattractive. Currently extraction from the plant, *Artemisia annua*, is the only viable method of obtaining artemisinin, with cultivation and re-manufacturing taking from 8-11 months (World Health Organization, 2005). Optimizing the production of this terpenoid biologically is therefore of great interest (World Health Organization, 2005).

1.2 Production of artemisinin

Considerable work aimed at optimizing artemisinin production has been done including breeding, cultivation studies, photohormone studies, media matrix analysis, viral transformations, biochemical pathway analysis and extraction optimization (Abdin et al., 2003; Gelder et al., 1997; Weathers et al., 1994, 1997, 2005). Overall the breeding of plants, manipulation of growth con-

ditions, growth media, and cell culture have been regarded as unsuccessful and further genetic engineering has been recommended (Abdin et al., 2003; World Health Organization, 2005). A review by Abdin et al. (2003) cites several transformation attempts, the best results increasing yields 2-3 times their normal level. Production levels in high yielding whole plants average around 0.5% with the highest being at 1.1% in a particular clone (Abdin et al., 2003). Currently one type of transformed hairy root clone has been at least partially optimized to produce higher artemisinin yielding *Artemisia annua* (Abdin et al., 2003; Weathers et al., 1997). Still, a major breakthrough in production yields is yet to be had, thus further study of the biochemical pathways is necessary.

1.3 Transformed *Artemisia annua* roots lose productivity over time

Although originally a promising source of artemisinin production, hairy root clones maintained in culture for greater than 10 years have lost their ability to make artemisinin (Table 1). This is not surprising considering that many *in vitro* cultures when maintained for many generations are known to become “habituated” and slowly lose phenotypic characteristics (Street et al., 1977a). This possibly suggests that some crucial genes are slowly being silenced as selective pressures are removed during culture (Street et al., 1977b). Previous work has shown that the methylation of DNA can account for some decreases

Table 1: Data showing a decrease in artemisinin production with age as published by Kim (2001).

Culture medium used (age of culture at assay)	Artemisinin content ($\mu\text{g/g DW}$) ^a	Reference & Notes
<i>Gamborg's B5, 3% sucrose</i>		
14 days	250	Weathers et al., 1994
14 days	375	Weathers et al., 1996
14 days	128	McCoy and Weathers, 2000, unpublished
<i>Gamborg's B5, 3% sucrose with half calcium</i>		
14 days	0.8	Wobbe et al., 1998
15 days	negligible	Kim, 2001, PhD Thesis

^a *A. annua* hairy roots are 8% dry weight when grown in liquid media.

in gene expression in plants (Emani et al., 2002; Paszkowski and Whitham, 2001; Klöti et al., 2002). Further, some reports indicate that demethylating DNA can restore gene expression (Emani et al., 2002; Klöti et al., 2002). The use of 5-azacytidine (azaC) is currently one of the most actively studied DNA demethylation techniques and even promises to prevent cell maturation by blocking differentiation (Lelijak-Levanić et al., 2004). To understand the effects of 5-azacytidine, one must look at the process of DNA methylation.

1.4 DNA methylation in plants

Methylated DNA typically occurs around areas of DNA where cytosine-guanine, and cytosine-N-guanine (where N is any nucleotide) are found, and highly concentrated occurrences of these typically occur in the promoter region of a gene (Paszkowski and Whitham, 2001; Calladine et al., 2004). This naturally facilitates the binding of promoter proteins that in turn help with the binding of transcriptional enzymes (Calladine et al., 2004). Promoter regions that are hyper-methylated, however, can cause excessive binding which can actually block the transcriptional enzyme from attaching itself to the DNA thereby silencing the gene (Paszkowski and Whitham, 2001). Further, DNA methylation can cause alterations in the structure of chromatin by facilitating the binding of histone de-acetylase 1, which removes acetyl groups from lysine groups, altering histone binding (Meyer, 2000; Calladine et al., 2004). This can further interrupt the way that transcriptional proteins bind to DNA and affect gene expression (Meyer, 2000).

During DNA replication, methylation sites are generally conserved. This is because an enzyme, methyltransferase, preferentially binds to cytosine-guanine and cytosine-N-guanine sites that are only partially methylated (Doerfler, 1983; Paszkowski and Whitham, 2001) as shown in Figure 2. Methyltransferase binds to the region and methylates the 5' carbon of the unmethylated cytosine, changing it to 5-methylcytosine (Doerfler, 1983; Calladine et al., 2004). Although, cytosine and 5-azacytidine are chemically similar as seen in Fig. 3, 5-azacytidine acts on methylation by displacing the cytosine

nucleotide in DNA during replication (Doerfler, 1983). Methyltransferase is unable to methylate the aza group; the nitrogen has no remaining valences, and thus cannot accept any more bonds.

1.5 Phenotypic recovery though demethylation

There is some evidence that decreases in phenotypic expression of certain secondary metabolites can be restored and even increased by treating plants with DNA demethylators such as 5-azacytidine. For instance, the transformation of sorghum sometimes results in decreased production of phosphinothricin acetyltransferase (PAT), a secondary metabolite that gives the plant resistance to herbicides (Emani et al., 2002). In a study performed by Emani et al. (2002), use of low concentrations of 5-azacytidine ($20\mu M$) was shown to restore and even increase the phenotypic expression of PAT in transgene sorghum. A nearly identical study was also performed on rice by Klöti et al. (2002) yielding comparable results. Promises of secondary metabolite yields increasing with the incorporation of DNA methylation inhibitors such as 5-azacytidine, have even provoked a patent filing by Stafford and Morville (2002). Thus, it is reasonable to test whether or not 5-azacytidine will be able to stimulate expression of the secondary metabolite, artemisinin, in *Artemisia annua*.

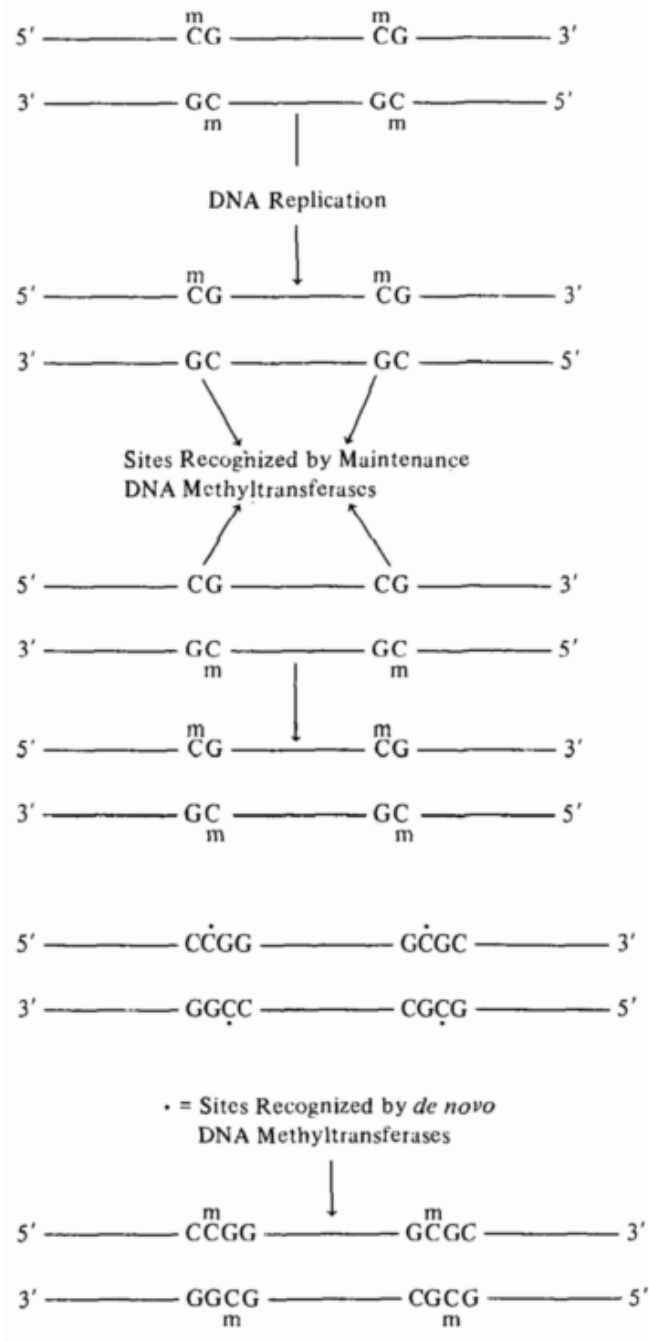


Figure 2: Methyltransferase acting on unmethylated cytosines as shown by Doerfler (1983).

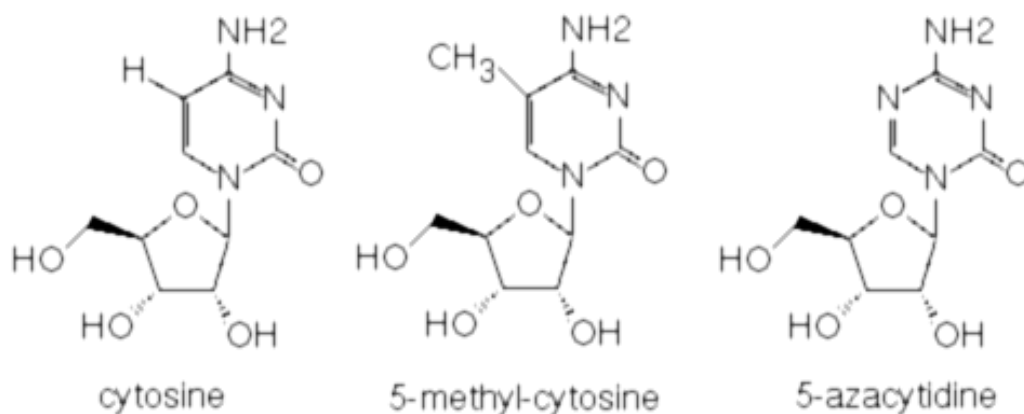


Figure 3: Cytosine and its analogues.

1.6 Past demethylation attempts in *Artemisia annua*

Earlier attempts by Woerdenbag et al. (1993) using 5-azacytidine on *Artemisia annua* shoots were unsuccessful in increasing artemisinin production. In that study, 5-azacytidine was incrementally introduced at 4 and 8 μM after the initial subculturing, until total final concentrations of 12 and 24 μM were reached halfway through the growth cycle (Woerdenbag et al., 1993). According to Woerdenbag et al. (1993) the addition of 5-azacytidine at these concentrations was too toxic for the cultures. However, most later studies involving other plants used 5-azacytidine at levels greater than or equal to 20 μM and reported no signs of toxicity and altered the phenotype of the plant being studied (Woerdenbag et al., 1993; Prakash and Kumar, 1997; Emani et al., 2002; Klöti et al., 2002; Horváth et al., 2003). Moreover, Emani et al. (2002) claimed that 5-azacytidine used on sorghum seeds was not toxic until a concentration near 310 μM was reached, nearly 15 times greater than Wo-

erdenbag et al. (1993) used in their work. Indeed, toxicity at the $20\mu M$ concentration is surprising as Prakash and Kumar (1997) were unable to observe any shoot induction in *Petunia* while using 5-azacytidine at the $5\mu M$ level. When used at the $20\mu M$ level, however, the induction of shoots was highly pronounced. Considering that shoot cultures like those used by Woerdenbag et al. (1993), generally perform quite differently in the lab than transformed hairy roots (Street et al., 1977b), it is reasonable to test 5-azacytidine at $20\mu M$ on low artemisinin producing *Artemisia annua* hairy root cultures.

2 Hypothesis & Objectives

2.1 Hypothesis

The YUT16 clone of *Artemisia annua* has effectively lost its ability to make artemisinin. This may be a result of the methylation of critical genes involved in artemisinin biosynthesis. Since 5-azacytidine is known to effect demethylation of DNA with some recovery of function, it is hypothesized that growing YUT16 hairy roots of *Artemisia annua* in 5-azacytidine will result in at least a partial recovery of artemisinin production.

2.2 Objective

2.2.1 To measure the effect of 5-azacytidine on the production of artemisinin in hairy root cultures of *Artemisia annua*.

2.2.2 To compare the level of demethylated DNA in roots with treatment of 5-azacytidine against roots without treatment.

3 Methods

3.1 Hairy root culture conditions

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

Experiments were performed using fourteen day old hairy roots cultures that were aseptically removed and blotted dry on a pre-sterilized maxi pad. Blotted roots were added to a tared, covered petri dish to yield a total of 0.4g fresh weight before being inoculated into 125mL flasks containing 50mL autoclaved Gamborg's B5 basal medium with 3% sucrose. Using a syringe, a freshly prepared solution of 12.21mg 5-azacytidine in 5mL dH₂O was filter sterilized (0.22µm) into a sterile plastic 30mL centrifuge tube. Using a fresh sterile 1mL syringe and needle, volumes of 0.05mL, 0.10mL and 0.25mL 5-azacytidine were added to shake flasks to respectively produce the 10µM, 20µM, and 50µM test media. Control cultures were made lacking any 5-azacytidine. Cultures were kept at 25°C, under continuous fluorescent white light, on an orbital shaker at 100 rpm for 14 days. After 14 days, culture medium was decanted from each flask. Freshly autoclaved media was prepared in 125mL flasks as before, and then was poured into the flasks con-

taining the hairy root cultures. A new solution of 12.21mg 5-azacytidine in 5mL dH₂O was filter sterilized and added in aliquots as previously described.

3.2 Extraction and analysis of artemisinin

After 2 complete subculturings (each 14 days) the roots were blotted dry and fresh weights obtained. Roots grown in 5-azacytidine did not produce enough fresh weigh to be analyzed on their own and therefore all three samples were pooled together. Two grams of fresh weigh from each control culture were used for each extraction. The roots were then placed in a test tube containing 1mL of toluene per gram of roots, the test tubes were capped, and artemisinin was extracted by sonicating in an ice bath for 30 minutes. The supernatant was removed and placed in corresponding labeled test tubes. The roots were re-extracted twice using the same ratios of toluene; supernatants were pooled and dried under nitrogen. Dried samples were stored at $-20^{\circ}C$ until ready for HPLC analysis.

Q260 HPLC analysis was performed according to Smith et al. (1997). Dried samples were resuspended in 100 μ L of methanol. Next, 400 μ L 0.2% (w/v) NaOH was added and the sample tubes were capped and placed in a heating block at 50 $^{\circ}C$ for 35 minutes. Upon removal from heat, tubes were placed in ice water then 400 μ L of 0.2 M acetic acid was added followed by another 100 μ L of methanol. Sample tubes were vortexed and then syringe filtered though a 0.22 μ m membrane (FP-200 Vericel) directly into a HPLC sample vial. HPLC analysis parameters were as follows:

- UV detector set at 260 nm;
- 15 cm Microsorb-MV C18 column, 4.6 mm i.d., 5 μ m beads with 100Å pore size,
- mobile phase of 55% 0.22 μ m filtered 0.01M phosphate buffer pH 7, 45% methanol,
- 1.0mL/min flow rate.

Quantitation was based on peak area of the sample injection compared against a 10 μ g/mL artemisinin standard.

3.3 Methylation studies & TLC analysis

An experimental procedure was developed to determine if 5-azacytidine had been incorporated into the DNA of *Artemisia annua* roots. Unfortunately, 5-azacytidine inhibited growth to the point that the procedure could not be used. Therefore, in order to thoroughly test the procedure as well as gain some insight on methylation patterns in *Artemisia annua*, DNA was extracted from *A. annua* T16 hairy roots and the shoots, roots of *A. annua* whole plants and analyzed for methylation.

3.3.1 Extraction of DNA from plant tissue

Extraction of genomic DNA from roots and shoots was performed by a modified version of the procedure described by Schuler (1989). Fully grown plants

were washed and blotted dry, a minimum of 10g of each tissue type was harvested, sterilized in a 5% Clorox solution for 15 min, rinsed 3 times in dH₂O, wrapped into aluminum foil, dipped into liquid nitrogen and crushed. The crushed tissue was then placed in a mortar and pestle with liquid nitrogen, ground to a fine powder, placed in a graduated cylinder, and 10 volumes of grinding buffer were added, prepared as described by Lilly et al. (2001): 0.45M sorbitol, 50 mM Tris buffer, pH 7.6, 5mM EDTA, 0.2% BSA, 1.0% polyvinylpyrrolidone-360, 0.025% spermidine, 0.025% spermine, and 1mL of β -mercaptoethanol (Tris buffer, pH 7.6 was prepared as described by Romagnano (2003)). Diethyldithiocarbonate was added until equivalent to 0.1M. The resulting solution was iced for 10 minutes, poured into a blender and pulsed on the highest setting for 5 seconds, allowed to settle, and pulsed on high again for another 5 minutes. Two layers of cheese cloth were placed over 50mL conical tubes and the resulting homogenate washed through with grinding buffer then transferred to centrifuge tubes.

The homogenate was then pelleted at 350 x *g* for 10 minutes, the supernatant discarded and pellet resuspended with gentle shaking in 5mL ice cold lysis buffer prepared as described by Ross et al. (1999) using 10mM NaCl, 1 mM EDTA, 10% glycerol, and 10 mM β -mercaptoethanol, 50mM of Tris-HCl buffer, pH 8.0. The DNA was then extracted by adding 2.5mL of chloroform and 2.5mL of phenol, gently shaking for 30 min and allowing to settle. A transfer pipet was then used to remove the top (aqueous) layer. The volume of the aqueous layer was measured and DNA was precipitated

by adding 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, gently mixing and then chilling at -80°C for 30 min. The solution was then centrifuged at $43,000 \times g$ for 10 min; the resulting pellet was resuspended in $900\mu\text{L}$ water, then reprecipitated by adding $100\mu\text{L}$ 2M NaCl and 2mL ethanol and holding at -80°C for at least 15 min. The reprecipitated pellet was then centrifuged again at $43,000 \times g$ for 10 min. To ensure the purity of the DNA sample, the OD at 280 nm of the supernatant was compared against a blank of $100\mu\text{L}$ 2M NaCl, $900\mu\text{L}$ water, and 2mL ethanol. The resuspension process was repeated until no absorbance at 280 nm was seen. The purified pellet was dissolved in 2mL water and the amount of DNA quantified by plotting the concentration and the OD at 260 nm of a water blank and solutions containing $0.1\text{mg}/\text{mL}$ and $0.02\text{mg}/\text{mL}$ DNA. A best fit line was found with the equation $y = 0.0796x$ where y is mg/mL DNA, and x is absorbance and then used to calculate the concentrations of the samples relative to their OD. All DNA samples were held at -80°C between testing periods.

3.3.2 Analysis of DNA base methylation by TLC

DNA bases separated by TLC (Table 2) were detectable with UV light to a sensitivity of $5\mu\text{g}$ for each base. Thus, 1mL of quantitated samples were hydrolyzed in 80% formic acid at 145°C for 45 minutes then subsequently dried under nitrogen. Dried samples were resuspended in $80\mu\text{L}$ water and

Table 2: TLC parameters and TLC sensitivity under short and long wave ultraviolet light.

Base	TLC Sensitivity	RF x100	Visibility		Preferred Solvent
			SUV	LUV	
adenine	5 μ g	67	✓		methanol
thymine	5 μ g	76	✓		hot methanol
guanine	5 μ g	18		✓	weak acid & hot ethanol
cytosine	5 μ g	24/47	✓	✓	hot methanol
5-methylcytosine	5 μ g	36/47	✓	✓	methanol

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

20 μ L formic acid and loaded onto a glass backed silica gel 60 TLC plate along with 0.05 mg of the standards cytosine and 5-methylcytosine. TLC analysis was performed using a chloroform:methanol:ammonium hydroxide (90:30:1) mobile phase. The plates were analyzed under long and short wave UV; locations corresponding to 5-azacytidine were circled in pencil, individually scraped from the plate and resuspended in 1.2mL of water. Plate scrapings were then vortexed and sonicated, then microfuged for 3 min. The amount of 5-methylcytosine in each supernatant was found by comparing the OD at 260 nm against a water blank and the 5-azacytidine standard scraping supernatant. This was then divided by the original DNA sample concentration to obtain the percent of DNA methylation in each tissue.

3.4 Data analysis

Shake flask experiments were replicated in triplicate. Due to a limited sample size all root growth and artemisinin production data collected will be subject to non-parametric statistical tests whenever possible. All data were matched against a control, so the Mann-Whitney Wilcoxon (Petruccelli et al., 1999a) and Friedman (Petruccelli et al., 1999b) tests were used to calculate any 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.

4 Results and Discussion

4.1 Effects of 5-azacytidine on growth and artemisinin production in *Artemisia annua* hairy roots

This experiment was based on the hypothesis that incorporation of 5-azacytidine into the hairy roots of *Artemisia annua* would result in increased artemisinin production. However, addition of 5-azacytidine to cultures significantly decreased the growth of *Artemisia annua* hairy roots (Fig. 4). Further, it appears that 5-azacytidine may have decreased overall artemisinin production in the roots (Fig. 5). However, these data are not statically significant mainly because the replicate 5-azacytidine root samples had to be pooled in order to do the HPLC analysis of artemisinin. Nonetheless, these data suggest that there is no increase in artemisinin production even with the addition of increasing amounts of 5-azacytidine (Fig. 4). These results correspond to those observed by Woerdenbag et al. (1993) in cultured shoots of *Artemisia annua*.

4.2 Toxicity of 5-azacytidine in *Artemisia annua* hairy roots

Woerdenbag et al. (1993) claimed the lack of increase in artemisinin production in shoots was because the 5-azacytidine was too toxic at the $20\mu M$ and $50\mu M$ levels. In contrast the data in Figures 4 and 6 show that 5-azacytidine

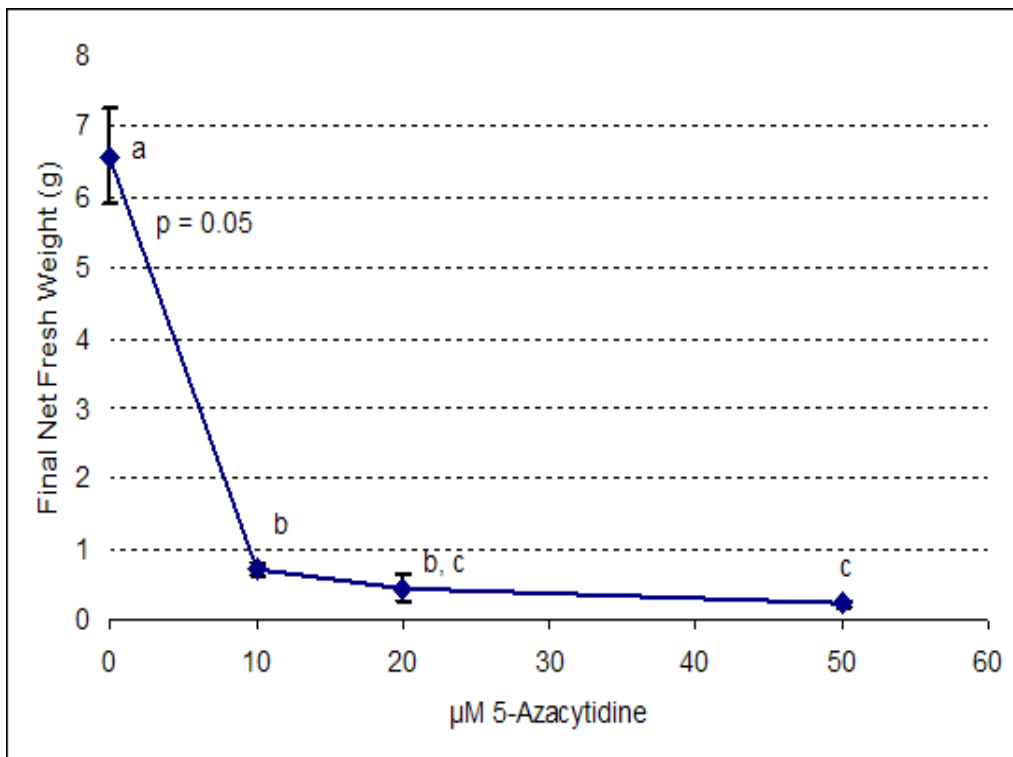


Figure 4: Root growth is inhibited by 5-azacytidine.

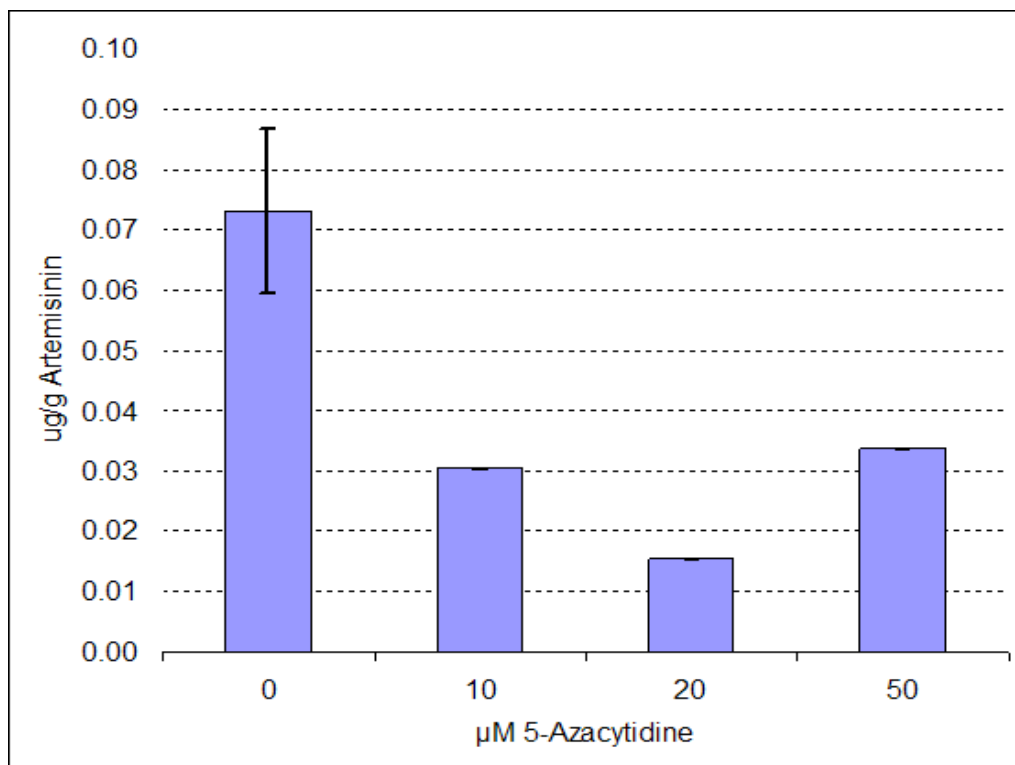


Figure 5: Artemisinin decreased in roots treated with 5-azacytidine.

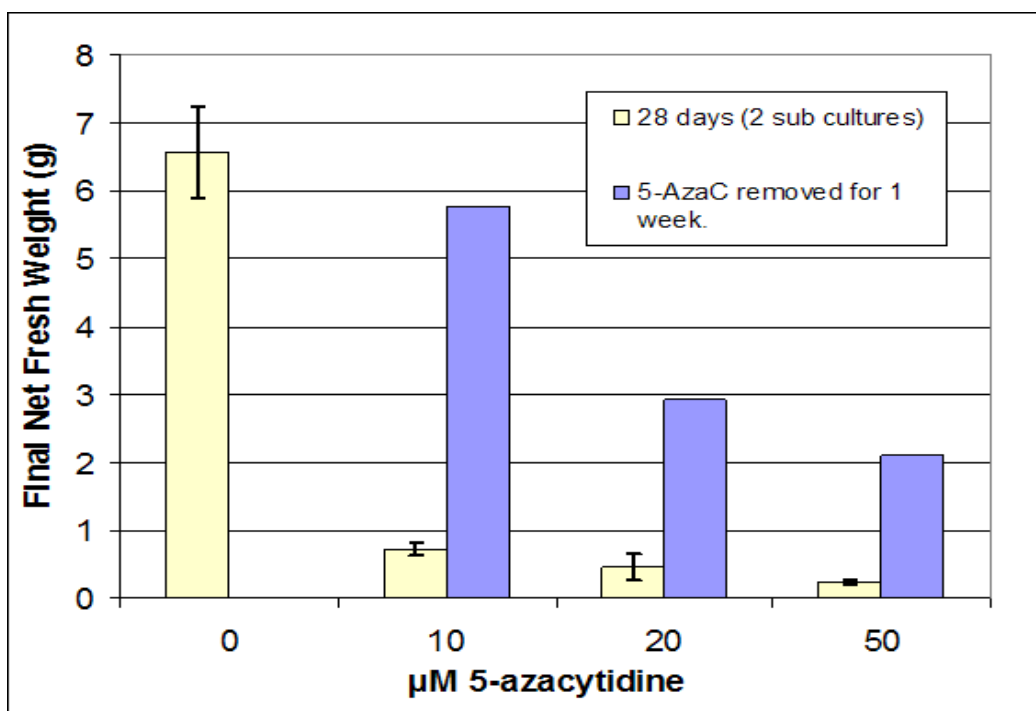


Figure 6: Root growth restored after removal of 5-azacytidine.

appears to be mainly a growth inhibitor rather than a plant toxin. Roots grown in the presence of 5-azacytidine grew normally when 5-azacytidine was removed (Fig. 6). These data were comprised of single samples, one at each concentration of 5-azacytidine and thus are not statistically significant.

4.3 Use of TLC for analysis of DNA methylation

Formic acid hydrolysis of DNA combined with TLC analysis is an effective way to separate the four major bases adenine, cytosine, guanine and thymine (Issaq et al., 1977). While TLC analysis is proven to separate cyto-

sine from 5-methylcytosine, the use of formic acid for methylation sensitive hydrolysis still remains questionable as methylated cytosines almost always become demethylated when hydrolyzed with stronger acids (Singer et al., 1977). Therefore, separation of nucleic 5-methylcytosine from cytosine can be challenging. When standard solutions of bases were assayed, they completely separated when visualized under shortwave UV light (Fig 7). Expected migrations are shown in Table 2. When the stock DNA samples hydrolyzed by formic acid were resolved, the four major bases appeared without indication of any methylated cytosine (Fig 7). However, lack of visible methylated cytosine may be a result of the technique used to purify the stock DNA. The amount of DNA extracted from hairy roots of *Artemisia annua* treated with 5-azacytidine was too small to be analyzed by TLC and did not resolve; a study on methylation between other related tissues was therefore performed.

4.4 Comparison of hydrolyzed DNA extract from transformed roots and normal roots and shoots

DNA extracted from roots, shoots and hairy roots of *Artemisia annua* was acid hydrolyzed using formic acid; the relative base composition was analyzed by TLC (Fig. 8). While there is a difference in the RF values from the previous plate, the standards that were run along with the samples to confirm the location of the bases. Differences in relative mobility are likely attributed to a switch in TLC plate manufacturer. Thus, the density or pH of the silica

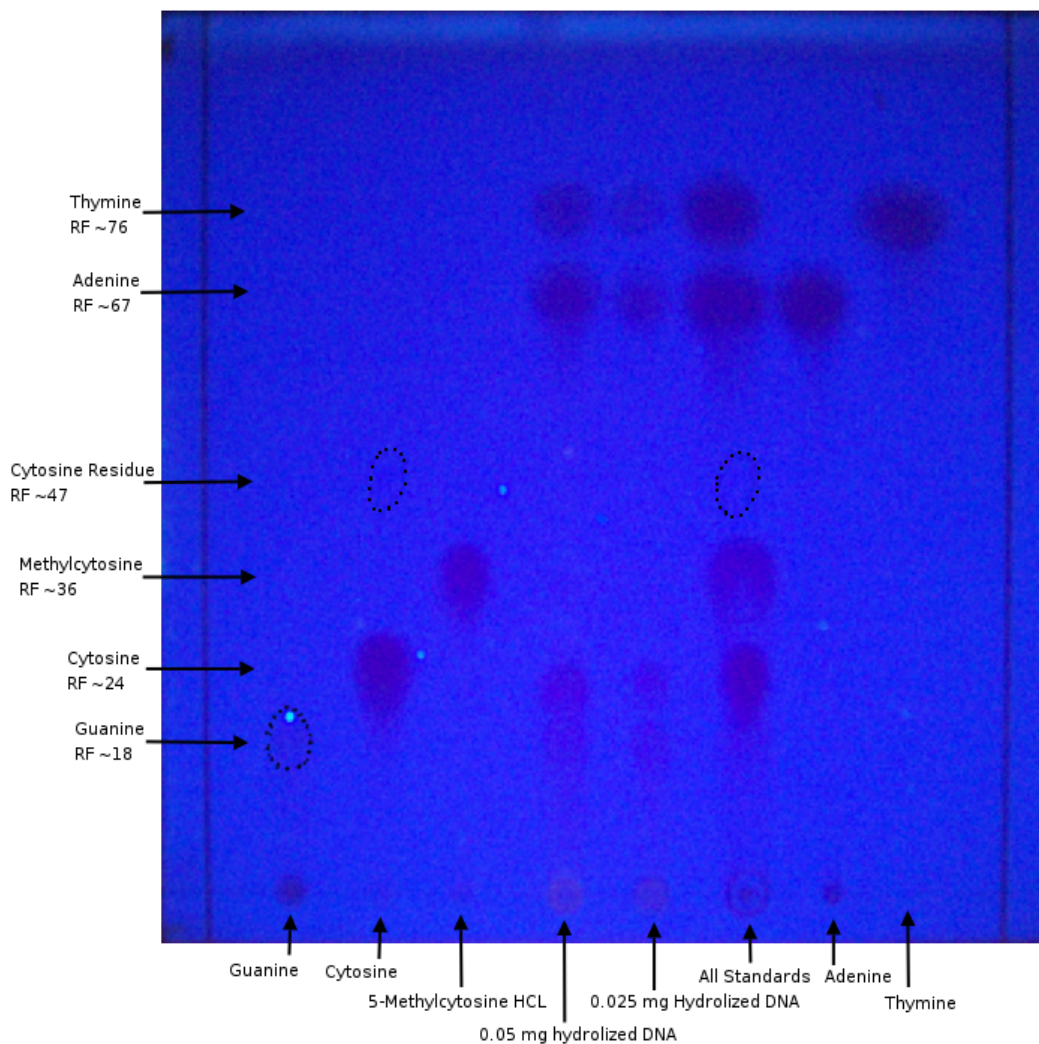


Figure 7: TLC of acid hydrolyzed DNA at UV 260 nm using a chloroform:methanol:ammonium hydroxide (90:30:1) mobile phase. RF refers to relative mobility x100 when compared to solvent front.

gel plate could be causing the cytosine and methylcytosine bases to run together and shift their RF values.

The relative percent of 5-methylcytosine in roots, shoots and hairy roots with respect to the initial amount of spotted hydrolyzed DNA is shown on a TLC plate (Fig. 8). When TLC plate scrapings of 5-methylcytosine were subsequently quantitated using spectroscopy, shoots showed the lowest amount of methylation, while normal roots had the most; hairy roots had quantities between the other two (Fig. 9). It is well established that roots produce no artemisinin, but shoots produce a lot (Ferreira and Janick, 1996). There, thus, seems to be a correlation between a lower amount of methylation in the shoots and that tissue's ability to produce higher quantities of artemisinin compared to the greater amount of methylation in roots and hairy roots which produce lower amounts of artemisinin.

4.5 Conclusions and future work

Consistent with Woerdenbag et al. (1993), treating roots with 5-azacytidine is not an effective way to increase artemisinin production. In fact, as seen in Figure 5, treating roots with 5-azacytidine resulted in a decrease in artemisinin production. Root growth rate was measured as follows:

$$\frac{FW_f - FW_i}{FW_i \cdot Days} = growth$$

Growth rates of $0.41g\ FW/day$ and $0.43g\ FW/day$ for the $20\mu M$ and

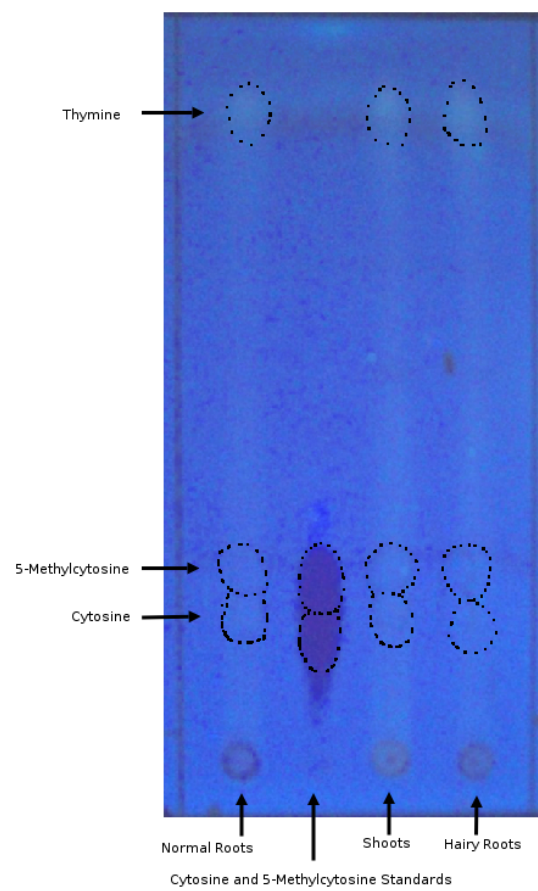


Figure 8: TLC analysis of acid hydrolyzed DNA from roots, shoots, and hairy roots using a chloroform:methanol:ammonium hydroxide (90:30:1) mobile phase.

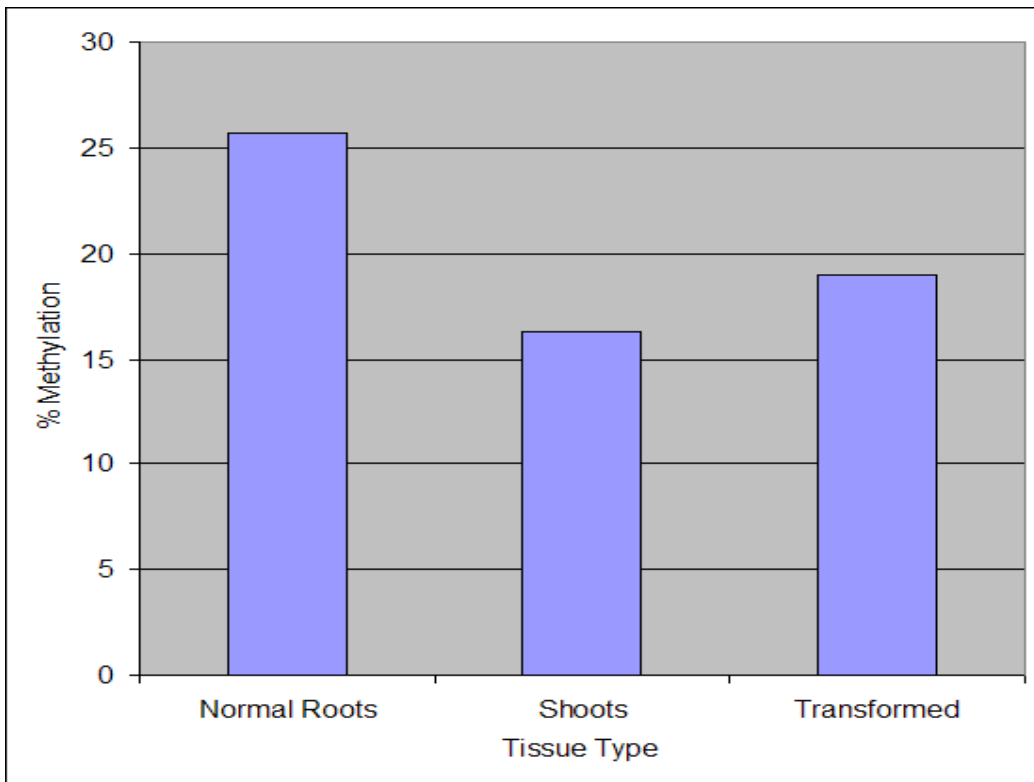


Figure 9: Percent 5-methylcytosine in hydrolyzed DNA samples of shoots, roots, and hairy roots calculated from quantitated TLC plate scrapings of 5-methylcytosine

50 μ M 5-azacytidine grown roots, respectively, were similar to the normal rate of 0.45gFW/day reported by Towler and Weathers (2003) for YUT16. In contrast the 10 μ M 5-azacytidine grown roots had a growth rate of 0.64gFW/day which was 1.4 times that of the YUT16 hairy roots reported by Towler and Weathers (2003). Roots in this study were, thus, shown to grow normally again after 5-azacytidine was removed from their media demonstrating that 5-azacytidine was not toxic to the roots. Indeed the original inhibition of growth shown in Figure 4 is more likely a result of an inhibition of root cell elongation, and not cell division. This result is in contrast to the work previously cited by Woerdenbag et al. (1993), which concluded that 5-azacytidine was not an effective way to increase the artemisinin production in shoots due to toxicity.

The extent of demethylation that occurred as a result of 5-azacytidine treatment in this study was not able to be determined mainly because there was too little tissue. Consequently, the role that DNA methylation plays in the phenotypic expression of artemisinin was not able to be determined with certainty. Further work on similar studies should consider choosing Methylation Sensitive Amplified Polymorphism (MSAP) after extraction of small quantities of DNA for methylation studies to ensure there is enough material to analyze. HPLC analysis may also prove advantageous over TLC for levels of methylation too small to visualize on TLC at UV 260 nm.

There appears to be an interesting difference in the methylation levels of normal roots, shoots, and transformed hairy roots. Yet the experiment

showing this was performed, however, with no replicates mainly because there was not enough time to grow several subcultures of roots in 5-azacytidine. To confirm this difference in methylation levels, future experiments to verify these effects would be helpful. Nonetheless the unreplicated data suggest that the tissues with the highest artemisinin yields, shoots (Ferreira and Janick, 1996), have significantly less methylation with respect to both types of roots (Fig. 9). Further, hairy roots, which currently still produce slightly more artemisinin than regular roots (Weathers et al., 2005), also seem to have slightly less methylated cytosine in comparison to regular roots (Fig. 9).

Demethylation of *Artemisia annua* hairy root DNA with other substrates still cannot be ruled out as a possible stimulation step. However, a study involving the addition and subsequent removal of sterile filtered 5-azacytidine to transformed hairy root cultures of *Artemisia annua* grown in Gamborg's B5 medium is still needed. If no increase in artemisinin is found, then it is probably reasonable to conclude that methylation has not played a role in the decrease in artemisinin production in the YUT16 clone of *Artemisia annua*. On the other hand, elicitors have been shown to reverse habituation; cytokinin-habituated isolates of tobacco were shown to reverse when cultured at 16°C instead of 26°C (Street et al., 1977a). Therefore a few tests on temperature and use of elicitors may prove interesting.

Currently, *in vitro* cultures do not seem to be a promising solution to world supply of artemisinin even despite optimization studies already performed (Weathers et al., 1997, 2005) because cultured *Artemisia annua* seems

to lose its ability to produce artemisinin over time (Kim, 2001) and isn't apparently recoverable by demethylation through the use of 5-azacytidine. Future experiments should therefore, more closely focus on the study of the pathways and mechanisms behind the biosynthesis of artemisinin. Ultimately isolation of all the genes in the production of artemisinin will allow for the transformation of these genes into better understood organisms where the production of artemisinin can be optimized.

References

- Abdin, M. Z., Israr, M., Rehman, R. U., and Jain, S. K. (2003). Artemisinin, a novel antimalarial drug: Biochemical and molecular approaches for enhanced production. *Planta Medica*, 69:289–299.
- Board on Global Health (2004). *Saving Lives, Buying Time: Economics of Malaria Drugs in an Age of Resistance*. National Academies of Sciences, Washington, D.C. pp 1-78.
- Calladine, C. R., Drew, H. R., Luisi, B. F., and Travers, A. A. (2004). *Understanding DNA, The Molecule & How It Works*. Elsevier Academic Press, third edition. pp 270-290.
- Doerfler, W. (1983). DNA methylation and gene activity. *Biochemistry Annual Reviews*, (52):93–124.
- Emani, C., Sunilkumar, G., and Rathore, K. S. (2002). Transgene silencing and reactivation in sorghum. *Plant Science*, (162):181–192.
- Ferreira, J. F. and Janick, J. (1996). Distribution of artemisinin in *Artemisia annua*. In Janick, J., editor, *Progress in new crops*, pages 579–584. ASHS Press, Arlington, V.A.
- Gamborg, O. L., Miller, R. A., and Ojima, K. (1968). Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research*, 50:151–158.
- Gelder, E. V., Vergauwe, A., and den Eeckhout, E. V. (1997). State of the art of the production of the antimalarial compound artemisinin in plants. *Plant Molecular Biology*, 33:199–209.
- Horváth, E., Szalai, G., Janda, T., Páldi, E., Rácz, I., and Lásztity, D. (2003). Effect of vernalization and 5-azacytidine on the methylation level of DNA in wheat (*Triticum aestivum* l. cv. martonvásár 15). *Plant Science*, (165):689–692.
- Issaq, H. J., Barr, E. W., and Zilinski, Jr., W. L. (1977). Separation of alkylated guanines, adenines, uracils and cytosines by thin-layer chromatography. *Journal of Chromatography*, (131):265–273.

- Kim, Y. (2001). *Assessment of Bioreactors for Transformed Root Cultures*. PhD thesis, Worcester Polytechnic Institute.
- Klöti, A., He, X., Potrykus, I., Hohn, T., and Fütterer, J. (2002). Tissue-specific silencing of a transgene in rice. *Proceedings of the National Academy of Sciences of the United States of America*, 99(16):10881–10886.
- Lelijak-Levanić, D., Bauer, N., Mihaljević, S., and Jelaska, S. (2004). Changes in DNA methylation during somatic embryogenesis in *Cucurbita pepo* L. *Plant Cell Reports*, 23:120–127.
- Lilly, J. W., Havey, M. J., Jackson, S. A., and Jiang, J. (2001). Cytogenomic analysis reveal the structural plasticity of the chloroplast genome in higher plants. *The Plant Cell*, 13:245–254.
- Meyer, P. (2000). Transcriptional transgene silencing and chromatin components. *Plant Molecular Biology*, 43:221–234.
- Paszkowski, J. and Whitham, S. A. (2001). Gene silencing and DNA methylation processes. *Current Opinion in Plant Biology*, 4:123–129.
- Petruccioli, J. D., Nandram, B., and Chen, M. (1999a). *Applied Statistics for Engineers and Scientists*. Prentice Hall. pp 657-662.
- Petruccioli, J. D., Nandram, B., and Chen, M. (1999b). *Applied Statistics for Engineers and Scientists*. Prentice Hall. pp 687-692.
- Prakash, A. P. and Kumar, P. P. (1997). Inhibition of shoot induction by 5-azacytidine and 5-aza-2'-deoxycytidine in *Petunia* involves DNA hypomethylation. *Plant Cell Reports*, 16:719–724.
- Romagnano, J. F. (2003). Aeration & mode of nutrient delivery affects growth of peas in a controlled environment. Master's thesis, Worcester Polytechnic Institute. pp 67.
- Ross, J. R., Nam, K. H., D'Auria, J. C., and Pichersky, E. (1999). S-adenosyl-l-methionine:salicylic acid carboxyl methyltransferase, an enzyme involved in floral scent production and plant defense represents a new class of plant methyltransferases. *Archives of Biochemistry and Biophysics*, 367(1):9–16.
- Schuler, M. A. (1989). *Plant Molecular Biology - Experiments*. Academic Press, Inc. pp 148-151.

- Singer, J., Stellwagen, R. H., Roberts-Ems, J., and Riggs, A. D. (1977). 5-methylcytosine content of rat hepatoma DNA substituted with bromodeoxyuridine. *Journal of Biological Chemistry*, 252(15):5509–5513.
- Smith, T. C., Weathers, P. J., and Cheetham, R. (1997). Effects of gibberellic acid on hairy root cultures of *Artemisia annua*: Growth and artemisinin production. *In Vitro Cell Developmental Biology*, 33:75–79.
- Sosnick Group University of Chicago (2005). Te buffer recipe. <http://sosnick.uchicago.edu/TE.html>.
- Stafford, A. and Morville, M. (2002). Manipulation of plant cell and tissue cultures. Patent: 20020081731.
- Street, H. E., Burnett, J. H., Baker, H. G., Beevers, H., and Whatley, F., editors (1977a). *Plant Tissue and Cell Culture*. University of California Press Berkeley and Blackwell Scientific Publications, second edition. pp 458-460.
- Street, H. E., Burnett, J. H., Baker, H. G., Beevers, H., and Whatley, F., editors (1977b). *Plant Tissue and Cell Culture*. University of California Press Berkeley and Blackwell Scientific Publications, second edition. pp 419.
- Towler, M. J. and Weathers, P. J. (2003). Adhesion of plant roots to poly-L-lysine coated polypropylene substrates. *Journal of Biotechnology*, 101:147–155.
- Weathers, P. J., Bunk, G., and McCoy, M. C. (2005). The effect of phytohormones on growth and artemisinin production in *Artemisia annua* hairy roots. *In Vitro Cellular & Developmental Biology - Plant*, 41:47–53.
- Weathers, P. J., Cheetham, R. D., and Teoh, K. (1994). Artemisinin production by transformed roots of *Artemisia annua*. *Biotechnology Letters*, 16(12):1281–1286.
- Weathers, P. J., Hemmavanh, D. D., Walcerz, D. B., Cheetham, R. D., and Smith, T. C. (1997). Interactive effects of nitrate and phosphate salts, sucrose, and inoculum culture age on growth and sesquiterpene production in *Artemisia annua* hairy root cultures. *In Vitro Cellular & Developmental Biology - Plant*, 33:306–312.

Woerdenbag, H. J., Luers, J. F., van Uden, W., Pras, N., Malingere, T. M., and Alfermann, A. W. (1993). Production of the new antimalarial drug artemisinin in shoot cultures of *Artemisia annua* L. *Plant Cell, Tissue and Organ Culture*, 32:247–257.

World Health Organization (2005). Facts on ACTs (artemisinin-based combination therapies) an update on recent progress in policy and access to treatment. http://www.who.int/malaria/cmc_upload/0/000/015/364/RBMInfosheet_9.htm.

Appendix A: Calculations

A.1 How much 5-AzaC is needed?

5-azacytidine has a molecular weight of 244.21. Therefore:

$$244.2 \frac{g}{L} = 1M$$

and

$$244.2 \frac{mg}{L} = 1mM$$

and

$$0.2442 \frac{mg}{L} = 1\mu M$$

The desired concentration is $20\mu M$.

$$4.884 \frac{mg}{L} = 20\mu M$$

Therefore, if one required $1L$ of $20\mu M$ media, one would only need $4.884mg$.

Our supply of $250mg$ should more than enough to meet our needs.

Most articles cite the use of either $50\mu M$, or $20\mu M$ concentrations of 5-azacytidine. It would be interesting to see if a lower concentration say $10\mu M$ would also be effective. A control will also be required to compare our results against.

Solutions with concentrations of $50\mu M$, $20\mu M$, $10\mu M$ and $0\mu M$ can be created easily by using media stock solutions equal to the highest and lowest

concentration 5-azacytidine required for the experiment ($50\mu M$ & $0\mu M$). By diluting the highest concentration media with the lowest concentration media, all the other concentrations can be obtained. For good statistical comparison, 3 flasks will required at each concentration. The total amount of each stock solution needed can be calculated:

Let $A = 50\mu M$ solution

Let $B = 0\mu M$ solution

	<u>A</u>	<u>B</u>
$3 \cdot 50mL \text{ of } A = 50\mu M$	= 150	0
$3 \cdot \left\{ \begin{array}{l} 20mL \text{ of } A \\ 30mL \text{ of } B \end{array} \right\} = 20\mu M$	= 60	90
$3 \cdot \left\{ \begin{array}{l} 10mL \text{ of } A \\ 40mL \text{ of } B \end{array} \right\} = 10\mu M$	= 30	120
$3 \cdot 50mL \text{ of } B = 0\mu M$	= 0	150
	<u>240</u>	<u>360</u>

We therefore need only $240mL$ of a $50\mu M$ media stock solution and $360mL$ of a control solution fore each generation that run according to the experiment above. A liter of $50\mu M$ media stock solution would therefore allow for 4 experimental generations. And since:

$$0.2442 \frac{mg}{L} = 1\mu M$$

A concentration of $50\mu M$ would only require $12.2mg$ as:

$$12.210 \frac{mg}{L} = 50\mu M$$

Appendix B: Dark Room Camera Setup

In order to document TLC plates under UV, picture exposure had to be set to 64 seconds. To avoid blurriness a way to position the camera above on a steady surface while the plates were held under UV light had to be derived(Fig 10, 11).



Figure 10: Top view of camera set up used to take long exposure TLC plate pictures under UV light



Figure 11: Side view of camera set up used to take long exposure TLC plate pictures under UV light