Artemisinin Yields in Chinese and Yugoslavian Strains of *Artemisia annua*

A Major Qualifying Project Report

submitted to the Faculty

of the

Worcester Polytechnic Institute

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

by

_____

Lujain Al-Sowaimel

April 30, 2009

_____

Professor Kristin Wobbe, MQP Advisor

Table of Contents

Table of Figures

List of tables

Abstract

The most effective treatment against malaria is artemisinin (AN), which is extracted from *Artemisia annua*. Considerable differences in the content of AN and its direct precursors are found in various *A. annua* plants of different geographical origins. From previous studies, AN yield in the Chinese (CH) strain is consistent and high compared to the Yugoslavian (YU) strain where the yield is lower and highly variable. Two possible causes of variability of AN content in the YU strain are reactive oxygen species (ROS) and AN precursor levels which are analyzed using a simple colorimetric method and liquid chromatography-mass spectrometry, respectively. There was no difference in H_2O_2 levels in either strain; the main cause of the low AN level in the YU strain is the precursor levels.

Acknowledgments

I would like to thank Professor Kristin Wobbe for giving me the opportunity to work on this project and for being my advisor.

A special thank to Patrick Arsenault for his patience, help and guidance throughout the course of this project. Also, I appreciated Patrick's assistance with the calculations part of this project, and for teaching me a lot of things in the lab.

I would like to thank Professor Pamela Weathers for using her lab equipment and materials.

I would like to thank Khanhvan Nguyen for letting me borrow some of her plants to do my experiments.

1. Introduction

Due to the increasing number of people afflicted with malaria every year and the development of resistance of different malaria strains to drugs, the need for a treatment that has the ability to overcome this disease is necessary. The most effective treatment against this disease is the drug called artemisinin (AN). This medicine is extracted from a plant called *Artemisia annua.* Unfortunately, *A. annua* produces low amount of AN, ranging from 0.1 to 1% of the plant dry weight, depending on the geographical origin of the plant (Wallaart et al., 1999). This plant was originally found in China, but it can be found in other places, such as Argentina, Bulgaria, France, Spain, USA, and the former Yugoslavia (Klayman, 1989, 1993). There is a tremendous difference in AN content in different strains of *A. annua* found in these different locations (Delabays et al. 1993; Woerdenbag et al. 1993). Therefore, understanding why certain strains produce more AN than other ones might help lead to an increase AN level. In a variety of studies, AN yield in the Chinese (CH) strain is consistent and high compared to the Yugoslavian (YU) strain, where the AN yield is lower and highly variable. Therefore, the main goal of this project is to determine the possible causes of the variability of the AN content in the YU strain by comparing and contrasting it to the CH strain of *A. annua.*

2. Background Information

2.1 Artemisia annua and Artemisinin

Artemisia annua is a plant that belongs to the Asteraceae family (Figure 1). This plant is single stemmed with alternate branches reaching more than 2 meters. This plant was originally found in China, but it can be grown in other places such as Argentina, Bulgaria, France, Spain, USA, and former Yugoslavia (Klayman, 1989, 1993). From *A. annua* an important compound is derived; this compound is called artemisinin (AN).

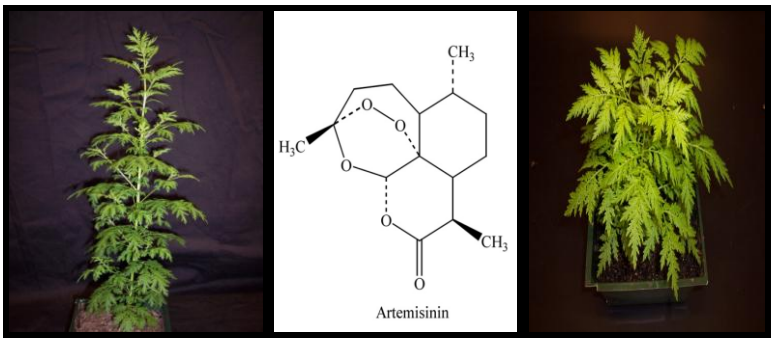

Figure 1: A Yugoslavian strain of *Artemisia annua* plant on the left; the chemical structure of artemisinin in the middle taken from Dhingra et al., 1999, and on the right a Chinese strain of *Artemisia annua*

AN is one of the secondary metabolites in *A. annua* (Bhandari et al., 2005). It is a sesquiterpene lactone with an endoperoxide bridge (Figure 1). AN can be purified from the aerial parts of *A. annua* (Webster et al., 1994). Roots and pollen were found to be AN deficient, but AN is present in main stems, side stems, leaves, and flowers of *A. annua* (Ferreira, 2004). AN is synthesized and sequestered in organs called glandular trichomes in *A. annua* plants (Figure 2). Glandular trichomes are composed of basal, stalk and three secretory pairs of cells (Covello et al., 2007).

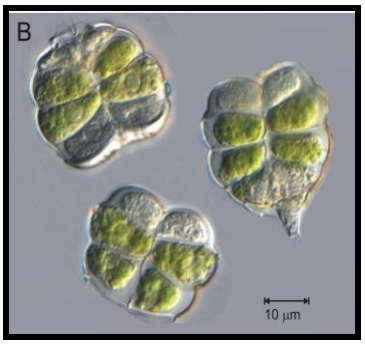

Figure 2: Glandular trichome structure taken from Covello et al., 2007

2.2 Therapeutic Aspects of Artemisinin

AN has many therapeutic benefits and uses. For example, *A. annua* has been used in China for colds and fevers since 340 AD. The first isolation of AN as the therapeutic entity in *A.annua* was done by Chinese scientists in 1972 (Dalrymple, 2006). AN has played several roles in treating various diseases, most importantly malaria, which will be discussed in more detail in the following section.

2.2.1 Artemisinin Use Against Malaria

Malaria is caused by the protozoa *Plasmodium* that is carried by a mosquito of the genus *Anopheles* (Bouwmeester et al., 2006). In 2006, there were 247 million malaria cases that led to nearly 881 000 deaths (World Health Organization, 2009). Malaria is more prevalent in tropical countries. The victims of this disease are most often children under the age of five; these children tend to live in the sub-Saharan African countries (van Agtmael et al., 1999). It was estimated that about 80-90% of the world's malaria is in Africa (van Agtmael et al., 1999). The second highest incidence of malaria is in South East Asia (Kumar et al., 2005). Each year overall incidence of

malaria continues to be about 500 million cases, which represents 350 million in the African region and 125 million in the South East Asian region (Kumar et al., 2005).

Traditionally malaria has been treated using different drugs such as chloroquine, quinine, mefloquine, and primaquine and with antifolates such as Fansidar (sulfadoxinepyrimethamine). Unfortunately, some malaria parasites have become resistant to these drugs (WHO, 2003). The main cause of this resistance is a genetic mutation in malarial parasites (Olliaro et al., 2004). Resistant cases can also occur when AN is given in short course monotherapy regimens (van Agtmael et al., 1999). Thus, the use of combination therapies with formulations including AN is the optimal solution to treat malaria (Kumar et al., 2005). This method is called artemisinin-based combination therapies "ACT". In ACT, AN kills most of the parasites rapidly, and what remains is killed by the combined drug (Olliaro et al., 2004). Examples of the combined drugs are chloroquine and sulfdoxime/pyrimethamine (Covello et al., 2007). ACT is considered the most successful therapy because of the absence under these regimens of any recorded cases of artemisinin-resistant malaria (WHO, 2003).

2.2.2 Artemisinin Mode of Action in Treating Malaria

The unique chemical structure of AN enables it to be a potent drug against malaria. The part of AN that is crucial in destroying a malaria parasite is a peroxide within a 1, 2, 4 trioxane configuration. The presence of this moiety in AN has led to several hypothesized mechanisms by which this drug works as an anti-malarial agent. Most of these mechanisms were based on understanding peroxide chemistry and chemical behavior of AN (Krishna et al., 2006).

One of these mechanisms propose that the destruction of malaria parasites by AN is based on the heme-mediated decomposition of the endoperoxide bridge to produce carbon-

centered free radicals (Meshnick et al., 2002). Unlike other oxidant drugs where it is necessary to react with oxygen to produce large quantities of oxygen-containing free radicals, AN itself is converted into a free radical in a reaction catalyzed by iron (Dhingra et al., 1999). The mechanism can be summarized in the following two steps. In the first step, AN is activated by an iron molecule, so free radicals are formed. Second, these free radicals work as electrophilic or alkylating intermediates damaging specific malarial membrane-associated proteins that are critical for the survival of *P.falciparum* (Meshnick et al., 1996).

A second mechanism by which AN destroys malaria parasites was recently proposed. This hypothesized mechanism is also based on free radical mechanism, but this time the free radicals specifically damage PfATP6, which is a sarco-endoplasmic reticulum Ca^{2+} -ATPase (SERCA) of *P. falciparum* (Eckstein-Ludwig et al., 2003). SERCA plays an important role in reducing cytosolic free calcium concentration by concentrating calcium into membrane-bound stores. This action is essential for cellular survival, specifically for intracellular signaling (Haynes et al., 2004). AN molecules show discrete specificity for SERCA of malarial PfATP6 as opposed to mammalian pumps. The detail of this mechanism is still under study (Haynes et al., 2004).

2.2.3 Artemisinin and other diseases

AN and its derivatives were shown to be therapeutic against schistosomiasis disease caused by the protozoan species *Schistosoma japonicum, S.mansoni,* and *S.haematobium*. This disease is known to cause about 1.5 million disabilities each year (Shuhua et al., 2002). AN is active against other protozoan infectious diseases such as leishmaniasis. In addition, AN can prove lethal to cancer cell lines, fungi, and bacteria (Kumar et al., 2005). Also, it has been used

to eliminate necrotic material from the body such as from wounds, intestines of people who have ulcerative colitis, and from Crohn's disease (Rowen, 2002).

2.3 Artemisnin Biosynthesis Pathway and Aretmisnin Production

It can be seen that AN can play a role in treating malaria and other diseases, including cancer. Unfortunately, an average *A. annua* plant produces little AN, about 0.01-0.8 % of the plant mass. Because neither total nor semi-synthesis is economically feasible (Abdin et al. 2003), the only source of AN is the plant that produces it (Bouwameester et al., 2006). Researchers have tried to enhance AN yields in different ways. One strategy is to understand the biosynthetic pathway of AN, including the enzymes that catalyze each step leading to AN and the factors that might increase the yield of AN.

2.3.1 IPP and DMAPP Biosynthesis

AN falls under the family of terpenoids. The primary precursor of terpenoids is isopentenyl diphosphate (IPP) and its isomer dimethyllallyl diphospahte (DMAPP). There are two pathways in which IPP is synthesized. One occurs in the cytosol, hence called the cytosolic mevalonic acid (MVA) pathway, and the other one occurs in the plastid, thus called the plastidial methylerythritol phosphate (MEP) pathway (Croteau et al., 2000).

In the MVA pathway, IPP is derived from acetyl CoA. The conversion of 3-hydroxyl-3-methylglutaryl CoA (HMG-CoA) into mevalonic acid is catalyzed by an enzyme called HMG-CoA reductase. For the MEP pathway, IPP originates from the condensation of pyruvate with D-glyceraldehyde-3-phosphate (Croteau et al., 2000).This condensation reaction is catalyzed by the enzyme deoxy-D-xylulose-5-phosphate synthase (DXS), which represents the first step of the

MEP pathway leading to the product 1-deoxy-D-xylulose-5-phosphate. IPP can be exchanged between the two compartments, the cytosol and plastid (Mahmoud et al., 2001).

A key enzyme responsible for catalyzing the step that produces many sesquiterpenes, including AN, is farnesyl diphosphate sythase (FPPase). This enzyme catalyzes the condensation of the C_5 substrate DMAPP with two molecules of IPP to the C_{15} product farnesyl diphosphate (FPP). FPP can then be cycled to sesquiterpene by a variety of sesquiterpene cyclase or go on to form sterols.

2.3.2 Artemisinin Biosynthesis Pathway

The committed step of AN synthesis is the cyclization of farnesyl diphosphate (FDP) into amorpha-4,11-diene by the enzyme amorphadiene synthease (ADS) (Figure 3). Then formation of artemisinic alcohol is catalyzed by the enzyme cytochrome p450 (CYP 71AV1). Artemisinic alcohol is oxidized by either CYP 71AV1 or artemisinic alcohol dehydrogenase into artemisinic aldehyde (Bouwameester et al., 1999). Artemisinic aldehyde has two fates, and each reaction is catalyzed via a different enzyme as shown in Figure 3. CYP 71AV1 action leads to the formation of artemisinic acid (AA), and artemisinic aldehyde hydrogenase (DBR2) activity leads to the formation dihydroartemisinic aldehyde. Dihydroartemisinic aldehyde forms dihydroartemisinic acid (DHAA) in a reaction step catalyzed by the enzyme dihydroartemisinic aldehyde dehydrogenase (AldH1). DHAA has only one possible route in the biochemical pathway. This route leads to the formation of AN in a hypothesized non-enzymatic photo-oxidative step. On the other hand, AA leads to the formation of arteannuin B (Zhang et al., 2008).

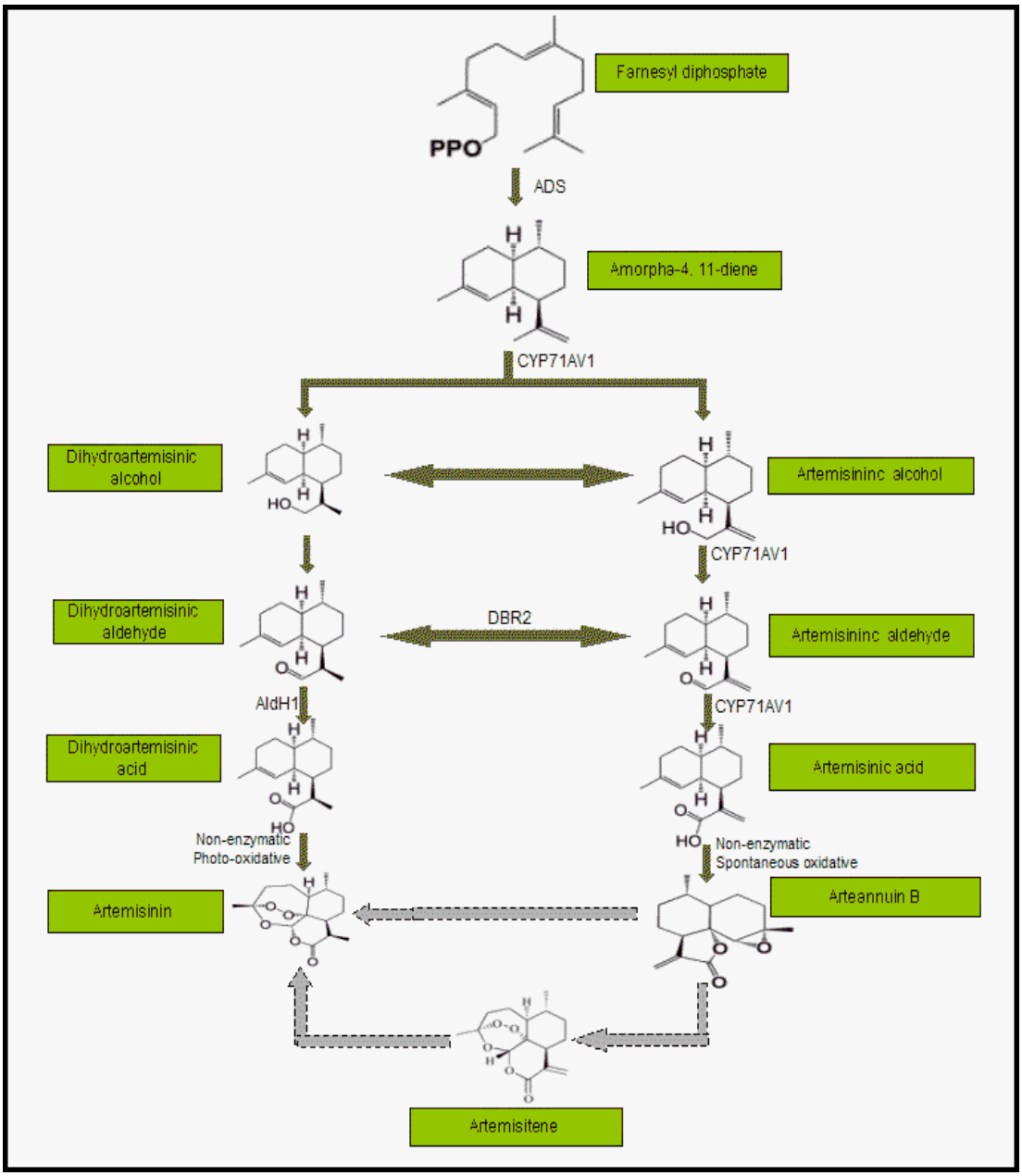

Figure 3: Artemisinin biosynthesis pathway adopted from Weathers et al., 2006, Zhang et al., 2008 and Wallaart et al., 1999

As stated in the introduction, the main goal of this project is to determine the possible causes of the variability in AN content in the YU strain as opposed to the CH strain of *A. annau*. This might be explained by the differences in precursor levels specifically, DHAA and AA. In

the CH strain, there might be more DHAA than AA, which would guarantee the production of AN. In the YU strain, the AN level might vary as the result of the presence of more AA than DHAA, which leads to the formation of AB. Some works suggested that the metabolites AB and, artemisitene are also precursors of AN (Zhang et al., 2008).

2.5 Reactive Oxygen Species and Artemisnin Production

In the last step of artemisinin biosynthesis, it is hypothesized that DHAA is converted into AN via a non-enzymatic step, which involves reactive oxygen species (ROS). It was proposed that dihydroartemisinic acid protects the plants by reacting with ROS yielding AN as a stable end-product (Wallaart et al., 1999).Therefore, another cause that might be responsible for the variability of AN content in the YU stain is the amount of ROS. Thus, it is important to understand what ROS are, learn some examples and sources, and comprehend their major roles in the bio-chemistry of plants. In this section, an overview of ROS will be given.

ROS are highly responsive reduced oxygen molecules. Some examples of ROS include singlet oxygen, superoxide anion, hydrogen peroxide, and hydroxyl radical. All these molecules share common properties in being highly reactive, toxic, and capable of causing oxidative damages to the cells (Asada et al., 1987).

Based on different studies, including pharmacological, molecular, and genetic studies, all suggest that the main source of ROS is a superoxide anion (O^{-}_{2}) generated by membrane-bound NADPH oxidase (Lamb et al., 1997). The major organelles responsible for the high production of ROS include the chloroplasts, mitochondria, and microbodies (Mittler et al., 2004). Usually under physiological steady-state circumstances, ROS are scavenged by different antioxidants. Therefore, there is a maintained level between the production and the scavenging of ROS, but in some cases

this balanced level gets disrupted. This interruption can be caused by environmental factors, which induce production of ROS. Consequently, ROS levels in the cells must be highly regulated (Pitzsche et al., 2006).

2.5.1 Reactive Oxygen Species Role in Plants

ROS have gained a bad reputation because in some cases they cause permanent damage and cell death. In fact, ROS are involved in a range of strategies to control diverse biological activities (Apel et al, 2004). ROS have an effect on cell signaling and gene expression. The success of ROS as signaling molecules implies that during the course of evolution, plants were able to attain a high degree of control over ROS toxicity. Thus, being able to manage ROS toxicity and enable ROS to act as a signaling molecule is all due to a large gene network (Mittler et al., 2004).This large gene network is known as "ROS gene network". It is responsible for directing a balanced level of ROS inside the cells, in addition to the duration, intensity, and subcellular localization of ROS signals (Mittler et al., 2004). In plants ROS play a significant role in the following biological processes: growth, development, stomata signaling, biotic and abiotic stress (Miller et al., 2007), cell cycle, programmed cell death (PCD) and hormone signaling (Mittler et al., 2004).

2.5.2 Oxidative Stress

As mentioned before, ROS can be produced via several means. In plants, ROS could be generated by activating different oxidases and peroxidases in response to environmental alterations (Apel et al., 2004). When the level of ROS production increases rapidly this might lead to an oxidative burst (Apostol et al., 1989). Oxidative burst "consists of a biphasic production of apoplastic ROS at the site of attempted invasion" (Zaniontto et al., 2006). Oxidative stress can be

initiated and triggered due to external conditions, which include biotic or abiotic stresses. Biotic stress might be caused by other organisms while abiotic stress occurs due an increase or decrease in the physical or chemical properties of the environment. Abiotic stress factors include high light, drought, cold, high temperature and mechanical stress (Apel et al., 2004). Ozone is found to be a direct precursor of ROS, and has the ability to induce PCD. Therefore, it is considered to be an abiotic inducer (Pitzschke, et al., 2006).

2.5.3 Hydrogen Peroxide

H_2O_2 is one of the ROS generated as a by-product in plant tissues during normal metabolism and under various stress conditions such as oxidative stress (Lu et al., 2008). H_2O_2 is mainly produced in chloroplasts and mitochondria through electron transport. In this process, first oxygen is reduced to superoxide and then dismuted into H_2O_2 spontaneously, or its conversion might be catalyzed by an enzyme called superoxide dismutase (SOD) (Asada 1999; Moller 2001). H_2O_2 can be produced by glycolate oxidase in peroxisomes (Noctor et al. 2002), membrane bound NADPH oxidase (Jiang et al., 2003), and oxalate oxidase (Hu et al. 2003). When plants are exposed to oxidative stress H_2O_2 acts as a signaling molecule. This is possible due to the fact that these molecules are small and diffuse over short distances, thus crossing the cellular membrane and act as a signaling molecule (Pitzsche et al., 2006). Thus, ROS metabolism in a specific compartment could change the ROS homeostasis of an adjacent compartment (Miller et al., 2007).

3. Hypothesis

The high and consistent level of AN in the CH strain of *A. annua*, as opposed to the low and variable level of AN in YU strain, is due to precursor levels, specifically DHAA, and a higher level of ROS facilitating the conversion of DHAA into AN.

4. Objectives

a. To quantify AN precursor levels in CH and YU strains using the method of liquid chromatography-mass spectrometry (LC-MS) with selective ion monitoring (SIM). The major metabolites this experiment will be focusing on are AN, AA, DHAA and AB.

b. To quantify ROS, specifically hydrogen peroxide, in YU and CH strains using a simple colorimetric method and relate the results to the amount of AN yields.

5. Methodology

5.1 Plant Growth Conditions

YU and CH seeds were grown in a liquid media for four days. After four days, seedlings emerged and then were transferred into Metro-Mix 360 soil and grown in a controlled environment inside a growth chamber with 16 hour days and 8 hour nights. The CH plants were maintained inside a growth chamber where the light intensity and temperature were 61µmol/m^2 and 28°C, respectively. YU plants were kept inside a growth chamber where the light intensity was 64.2µmol/m^2 and temperature 25°C, respectively. Leaves were harvested from both strains after four weeks.

5.2 Measuring Metabolites Level

LC-MS with selected ion monitoring SIM as developed by Patrick Arsenault was the technique used in this study for determination of the metabolite levels, specifically AN, DHAA, AA and AB in *A. annua*. LC-MS analyses were done using Aglient 1100 system equipped with an autosampler, a quaternary pump system, a photodiode array and multiple wavelength detector, a thermostated column compartment, a degasser, and Chemstation software.

5.2.1 Analytical Standards

An individual stock solution of each metabolite was prepared by dissolving each one in appropriate volume of toluene: 0.06 mg AN in 600 µl, 1.6 mg AA in 1600 µl, 1.4 mg of AB in 1400 µl, 0.05 DHAA in 500 µl.

5.2.2 Sample Preparation

Approximately 2 g of leaves from each of six plants were frozen in liquid nitrogen and ground separately in a mortar into fine powder. Then, 0.1 g of each plant tissue was placed into individual glass test tubes and 2 ml of toluene was added. Tubes were sonicated in an ice bath for 30 minutes. The tubes were removed and the supernatant was decanted into new tubes, while the plant pellets were resuspended in another fresh 2 ml of toluene. Tubes were sonicated again for 30 minutes and the resulted supernatant was added into the first supernatant.

The supernatant was then semi-purified using the technique of flash chromatography. A 3 ml column was packed with 0.5g of silica gel 60 Å was preconditioned with 2 ml of toluene. About 4 ml of plant tissue extract was loaded on the column, followed by washing with 2ml of toluene. Subsequently, the samples were eluted with 2x 0.5 ml of methanol. Vacuum was applied at the end to make sure that all metabolites were eluted. The 2 ml collected fractions were evaporated under nitrogen gas to dryness and resuspended in 1 ml of acetonitrile then vortexed. Finally, the 1 ml sample was filtered using a 0.2 µm syringe filter.

5.2.3 Sample Analysis Using Liquid Chromatography-Mass Spectrometry

The purified samples were run on the Agilent 1100 LC-MS with an ODS3 column. The mobile phase consisted of 75 % water (0.1% formic acid) 25% acetonitrile. The flow rate was set at 0.400 ml/min. Injection volume was 1.0 µl, the stop time was 32 minutes, and the post running time was 3 minutes. The heater temperature surrounding the LC/MS column was 35˚C. High-purity nitrogen (99.999%) was used as dry gas, the flow rate was 10.1 L/min, the capillary temperature was 350˚C, and helium was used as nebulizer at 40 psi. Table 1 shows the polarity of the ion optics in the ion transport while Table 2 shows SIM parameters.

Each sample was further separated in the LC-MS, and different peaks were generated. These peaks corresponded to various components in the sample mixture. To obtain qualitative information regarding the sample composition, peak positions were compared with analytical standards as discussed in section 5.2.1. Quantitative information was obtained from peak areas, and the standard curves were used to determine the amount of the metabolites

Signal number	Polarity
1	Positive
2	Negative
3	Negative
4	Positive

Table 1: Polarity of the ion optics in the ion transport

Signal number	Time (min)	Group Name	SIM	Frag-Ion	Gain mentor	Actual EMV
1	0.00	Group 1	283.20 284.20	70 70	1.0 ----	97 ----
2	0.00	Group 1	235.20 236.20	70 70	1.0 ----	97 ----
3	0.00	Group 1	233.20 234.20	70 70	1.0 ----	97 ----
4	0.00	Group 1	266.2 267.2	70 70	1.0 ----	97 ----

Table 2: SIM parameters

5.3 Hydrogen Peroxide Detection and Quantification

Hydrogen peroxide (H_2O_2) quantification was adopted from Zhou (et al., 2006). The principle of this technique is based on production of a stable red product upon the reaction of H_2O_2 with 4-aminoantipyrine and phenol in the presence of the enzyme peroxidase.

5.3.1 Sample Preparation

The leaves of six different plants from each CH and YU strain were frozen in liquid nitrogen and ground in a mortar with pestle. Once thoroughly ground, 0.5g of plant tissue was added into 15 ml conical tubes. To each tube, 5 ml of 5% trichloroacetic acid (TCA) solution and 0.25g of polyvinylpolypyrrolidone (PVPP) were added. Each tube was vortexed prior to centrifugation at 8,000 rpm for 20 minutes at 4˚C.

The supernatants were decanted into a plastic test tubes and the pH was adjusted to 8.4 using 2 M sodium hydroxide. Once the pH was adjusted, the solution was filtered using a 0.45 µm syringe filter. From the filtrate, two equal amounts of 1 ml aliquot were taken and put into separate glass tubes. To one aliquot of the original filtrate, 1.6 µl of catalase was added. Both aliquots were then kept at room temperature for 10 minutes. One ml of colorimetric reagent, which consisted of 10 mg of 4-aminoantipyrine, 10 mg of phenol, and 5 mg of peroxidase, dissolved in 50 ml of 100 mM acetic acid buffer (pH 5.6), was added to both aliquots. The resultant mixture was incubated for 10 minutes in a water bath at 30˚C. The absorbance was determined spectrophotometrically at 505 nm.

5.3.2 Constructing a Standard Curve

Five different concentrations of H_2O_2 were prepared using 0.88 M H_2O_2 stock solution. At the beginning 0.88 M H_2O_2 was 10x diluted in 5% TCA. Once it was diluted, 6.8 µl was added to 2ml diH_2O to reach a final concentration of 300 µM. A 2-fold serial dilution was performed achieving concentrations of 150 µM, 75 µM, 37.5 µM and 18.75 µM. One blank consisting of only 2ml of diH_2O was prepared. To the blank solution, 1.6 µl of catalase was added and kept for 10 minutes. Then, 1 ml of the colorimetric reagent was added to the six tubes

including the blank and incubated in a water bath for 10 minutes at 30˚C. The absorbance of each sample was taken at 505 nm. Using these absorbences, a graph of absorbance at 505 nm versus H_2O_2 concentration for each H_2O_2 standard was generated (Figure 5).

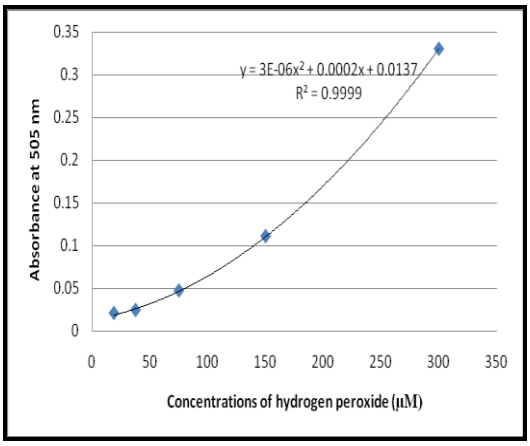

Figure 4: Standard curve of absorbance vs. hydrogen peroxide concentration.

5.4 Effect of Polyvinylpolypyrrolidone on Measuring Hydrogen Peroxide

This experiment was conducted to judge the correct amount of polyvinylpolypyrrolidone (PVPP) that should be added to the plant tissue extracts discussed in section 5.3.1. PVPP was added to the plant samples to eliminate interfering compounds such as phenol and ascorbic acid. These compounds must be removed because they might cause failure to detect H_2O_2 in the measuring system. In this method, various amount of PVPP and different concentrations of H_2O_2 stock solution (concentration 0.88 M) were used. Table 3 illustrates this experimental design.

Group number	Samples labeling	Volume of 5% TCA (ml)	Amount of PVPP (g)	$[H_2O_2]$ (µM)
1	**A**	5	0.25	300
	B	5	0.25	150
	C	5	0.25	37.5
2	**A**	5	0.5	300
	B	5	0.5	150
	C	5	0.5	37.5
3	**A**	5	1.0	300
	B	5	1.0	150
	C	5	1.0	37.5

Table 3: The relationship between various amounts of PVPP at different hydrogen peroxide concentrations

5.5 Superoxide Detection Method

Superoxide detection was adopted from a method developed by Fryer et al., 2001. In this method, nitroblue tetrazolium (NBT) was the dye used to detect superoxide. This dye has a pale yellow color, but once it reacts with superoxide a dark insoluble formazan compound is produced. This technique is qualitative only. *A. annua* leaves were infiltrated with 6 mM of NBT. Only one CH plant was used for this part of the experiment. A section of leaf on this plant was taken, and submerged inside a test tube filled with NBT solution. The tube was placed in a beaker covered with a larger beaker inside a bell jar that was connected to a vacuum pump. Figure 4 illustrates the vacuum infiltration set up. The air inside the beaker was drawn until bubbles formed on the leaf and stem surface. Once the solution started to bubble, the vacuum was released very rapidly.

Chlorophyll pigment was removed by placing the leaf in a beaker of 100 % boiling ethanol. Once the leaf turned white with some dark blue spots, it was examined under the light microscope. This experiment was repeated for multiple leaves on this plant.

Figure 5: Vacuum infiltration set up, viewed from the outside; detailed view from the inside

6. Results and Discussion

6.1 Precursor level quantification

To determine the possible causes of AN variation in the YU strains of *A. annua*, AN and its precursors were quantified using LC-MS. The four metabolites quantified were artemisinin, dihydroartemisinic acid, artemisinic acid, and arteannuin B. The quantification of the metabolite levels in the CH and YU strains of *A. annua* was based on the LC-MS peak area for each metabolite, and a standard curve was used for calculation. The detection and identification of the peaks were performed by comparison to known reference standards. AN, AA, and AB were quantified for 6 plants from each strain, CH and YU. Figure 6 shows the amount of AB in the CH (blue bar) and YU (red bar) strains. The amount of AB in the YU strain is 25X the amount of AB in the CH. Therefore, YU might follow, or emphasize, the AB pathway most of the time where it produces AB as its final product (Figure 3). Also, the variability of AN levels in the YU strain can be explained from the AN biosynthesis pathway. In this pathway, AB might not be always the final product, because it might go to form AN or artemisitene as shown in Figure 3.

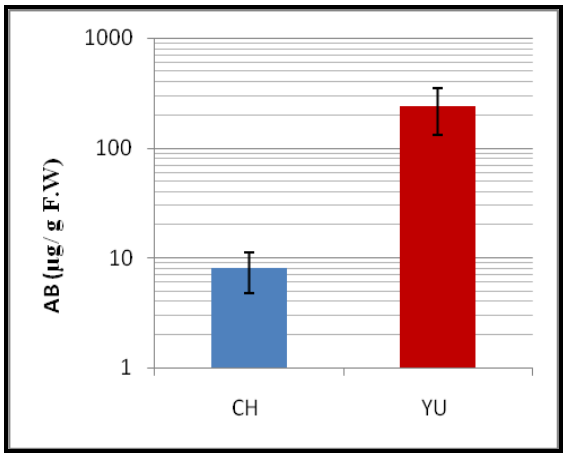

Figure 6: Comparison of the amount of arteannuin B in the CH and YU strains of *A. annua*

AN levels for the CH strain are 10X the amount of AN in the YU strain as shown in Figure 7. Therefore, the CH strain might follow the AN pathway most of the time. In this case, it might start from farnesyl diphosphate going directly through DHAA to AN. According to Wallaart et al. (1999), plants with a higher AN yield tend to have a higher DHAA yield. Unfortunately, we could not determine the relationship between DHAA and AN, since DHAA data were not available. Another possible explanation for the high AN production in the CH strain is that there might be a step that involves a highly efficient enzyme (the last hypothesized step in Figure 3 going from AB to AN directly), converting substantial amounts of AB to AN in the CH. On the other hand, in the YU strain, this enzyme is less efficient. Thus, a lot of AB accumulates instead of being converted to AN.

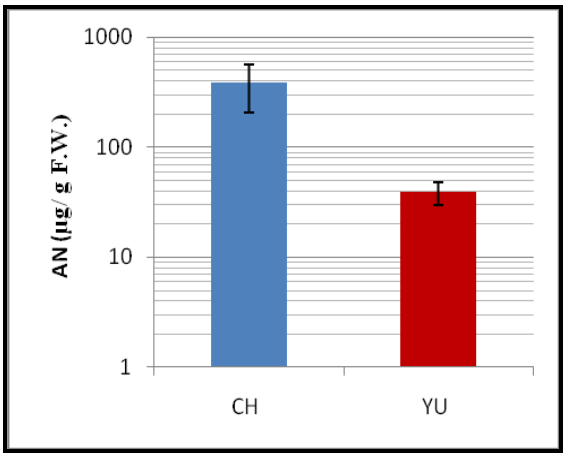

Figure 7: Comparison of the amount of artemisinin in the CH and YU strains of A. annua

Figure 8 shows that the amount of AA in the CH strain is 5X the amount of AA in the YU strain. This might be explained by the presence of reactive oxygen species (the quantification of these species is in the next section) up-regulating enzymes earlier in the biosynthetic pathway responsible for producing a lot of AA in the CH strain. AA is very high

relative to the other metabolites, including AN and AB, in both YU and CH strains. This is probably an indication that AA is the main precursor for both AN and AB in both strains.

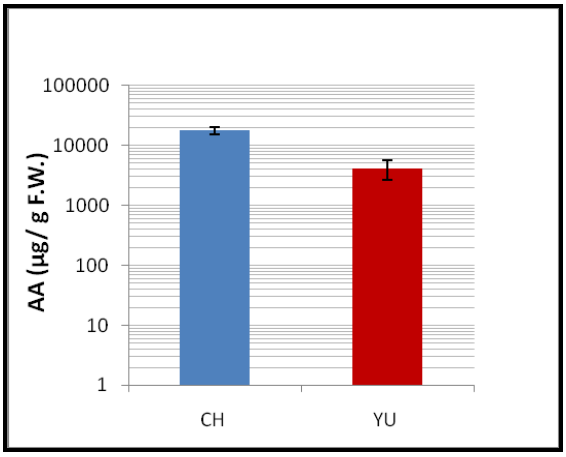

Figure 8: Comparison of the amount of artemisinic acid in the CH and YU strains of *A. annua*

6.2 Superoxide Detection Method

We hypothesized that the other possible cause of AN variation in YU strain was the presence of low concentrations of reactive oxygen species compared to the CH strain. The two ROS looked at were hydrogen peroxide and superoxide. A simple colorimetric method was used to quantify H_2O_2 in both strains, and superoxide was detected using a dye.

The dye nitroblue tetrazolium (NBT) has a pale yellow color, but once it reacts with superoxide, a dark insoluble formazan compound is produced. Figure 9 shows the staining area on one section of a CH leaf. The stained area corresponds to the presence of superoxide. This methodology to detect superoxide was not helpful because it only gave qualitative information and not quantitative results as shown. Therefore, the focus was shifted to quantify hydrogen peroxide and relate the amount of hydrogen peroxide to the overall amount of reactive oxygen species in the system.

Figure 9: NBT staining on a CH *A. annua* leaves. The blue staining on the leaf corresponds to the areas where superoxide is present

6.3 Effect of Polyvinylpolypyrrolidone on Measuring Hydrogen Peroxide

The simple colorimetric method used to quantify hydrogen peroxide involves the use of charcoal to remove of all compounds that might interfere with H_2O_2 detection. However, some H_2O_2 was also removed; this is also shown in Zhou et al. (2006). Then we looked at other compounds that have the ability to remove the interfering compounds from the assay without affecting H_2O_2 levels. Lu et al. (2008) found polyvinylpolypyrrolidone (PVPP) is an alternative solution to charcoal.

An experiment was performed to determine the correct amount of PVPP that should be added to the plant tissue extracts to eliminate interfering compounds, such as phenol and ascorbic acid. In this experiment, different amounts of PVPP were (0.25, 0.5 and 1 g) added to different concentrations of H_2O_2 stock solution (concentration 0.88 M). These concentrations were 300, 150, 75 µM.

Comparing the results generated using 0.25g and 0.5g PVPP, it can be seen that PVPP increases absorbance for group 2 samples, which contain 0.5g of PVPP, although the same H_2O_2

concentrations were used in groups 1 and 2; thus, the expected outcome was that the same H_2O_2 signal would be generated. From Figure 10, it is clear that the outcome from each group (1 and 2) was different. This implies that the amount of PVPP affects the signal H_2O_2. Since increasing the amount of PVPP affects the experimental results, only 0.25g of PVPP will be used to prevent this problem from happening while performing hydrogen peroxide quantification. Figure 10 also shows that 0.25g is preferable because there is a linear relationship between H_2O_2 absorbance and the presence of 0.25 PVPP; the square of the correlation coefficient is higher than that of group 2. The relationship between H_2O_2 absorbance and 0.5g PVPP is not linear and the correlation coefficient is lower. Group 3, which represents 1g of PVPP that was added to H_2O_2 samples, was ignored because the solution was too saturated; hence no liquid was generated for analysis. In conclusion, among the three groups, Group 1 was the optimal one.

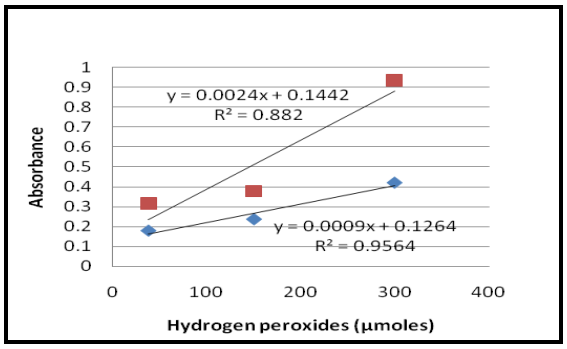

Figure 10: Effect of different amount of PVPP on measuring hydrogen peroxide

Key: ◆ 0.25 g PVPP group ■ 0.50 g PVPP group

6.4 Hydrogen peroxide quantification

Hydrogen peroxide was quanified using a simple colorimetric method. The method is based on hydrogen peroxide producing a stable red product upon the reaction with 4-aminoantipyrine and phenol in the presence of the enzyme peroxidase. The concentration of the product was determined spectrophotometrically at 505 nm and related to hydrogen peroxide. As

seen in Figure 11, the amount of hydrogen peroxide was not statistically different in the two strains.

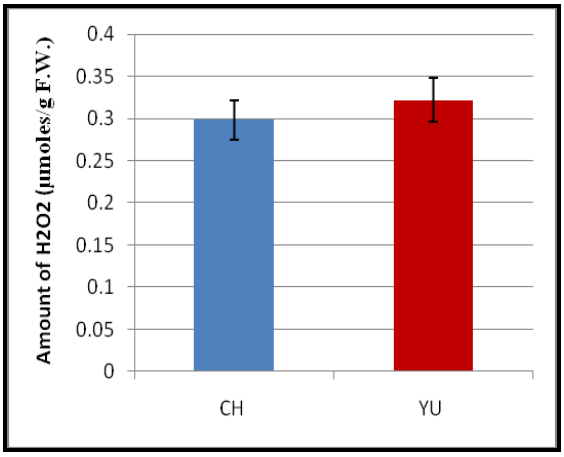

Figure 11: Comparison of the amount of hydrogen peroxide in the CH and YU strains of *A. annua*

To determine if there is a relationship between the amount of hydrogen peroxide and the amount of artemisinic acid in the YU strain, we tested several individual plants. 5 YU plants were harvested after four weeks; Tissue from each of five different plants was split into two samples. One was used to measure H_2O_2 levels as above; the other half was used to quantify metabolites via LC-MS. From Figure 12, it can be seen that there is a direct correlation between the amount of ROS (hydrogen peroxide) and the amount of artemsinic acid in 5 individual plants of YU strain. At higher amounts of H_2O_2, there is more AA. For example, from Figure 12, when the amount of hydrogen peroxide went from 0.29 to 0.35 µmoles/ g F.W., the amount of AA went from 3503.4 to 4550.8 µg/ g F.W. Thus, a linear relationship can be obtained with a square of the correlation coefficient at 0.6521. As explained before, this might happen because ROS up-regulate some enzymes responsible for the high production of AA. This data was not conclusive because of the small sample numbers.

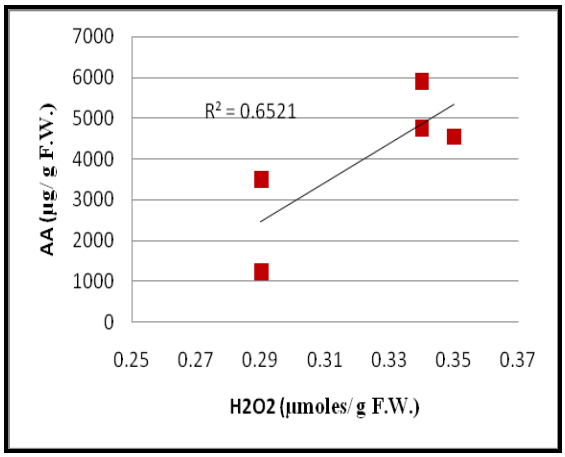

Figure 12: Correlation of hydrogen peroxide amount and artemisinic acid level in 5 plants of YU strain of *A. annua*

7. Conclusions and Future Work

There is an inverse relationship between AN and AB levels in both strains. When AB is low, AN is high and when AB is high, AN is low, which occurred in the CH and YU strains, respectively. Hence, it appears that YU follows the AB pathway and CH follows the AN pathway. ROS might be up-regulating some enzymes that may be responsible for high production of artemisinic acid in CH strain of *A. annua*. The levels of hydrogen peroxide in both strains (CH and YU) are the same and might have no affect on the variability of AN in the YU strains of *A. annua,* but there could be variations in the other ROS that could be influencing AN production in both strains.

Future work should include measuring the levels of DHAA to provide further information about the differences in AN production in YU and CH strains. The ROS data suggests that we should quantify reactive oxygen species other than H_2O_2, such as singlet oxygen, superoxide anion to further conclude if there are differences in ROS in the two strains. Also, future work will focus on the role of redox state in the regulation of AN biosynthesis. Since there is a balance between oxidants and antioxidants in the system, there might be no change in the overall oxidative stress. Therefore, amounts of oxidants and antioxidants must be measured and checked for their effects on AN production. If oxidants increase AN production then we need to inhibit antioxidants from functioning to increase AN production.

References

Abdin, M.Z.; Israr, M.; Rehman, R.U.; Jain, S.K. (2003) Artemisinin, a novel antimalarial drug: biochemical and molecular approaches for enhanced production. *Planta medica*, 69: 289-299.

Apel, K. and Hirt H. (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu Rev Plant Biol*, 55: 373-99

Apostol, I; Heinstein, P.F.; Low, P.S. (1989) Rapid stimulation of an oxidative burst during elicidation of cultured plant cells-Role in defense and signal transduction. *Plant physiology*, 90: 106-116

Asada K, Takahashi M (1987) Production and scavenging of active oxygen in chloroplasts. *In* DJ Kyle, CB Osmond, CJ Arntzen, eds, Photoinhibition. Elsevier, Amsterdam, pp 227–287

Asada, K. (1999) The water–water cycle in chloroplast: scavenging of active oxygens and dissipation of excess photons. *Annu Rev Plant Physiol Plant Mol Biol*, 50:601–639

Bhandari, P.; Gupta, A. P.; Singh, B.; Kaul, V. K. (2005) Simultaneous densitometric determination of artemisinin, artemisinic acid and arteannuin-B in Artemisia annua using reversed-phase thin layer chromatography. *Journal of Separation Science*, 28: 2288-2292

Bouwmeester, H.J.; Bertea, C.M., Kraker,J.W. d; Franssen, M.C.R.(2006) Reserach to improve artemisinin production for use in the preparation of anti-malaria drugs. *Medicinal and Aromatic Plants*: 275-290

Covello, P.S.; Keat, H.T.; Devin, R. P.; Darwin, W. R.; Goska, N. (2007) Functional genomics and the biosynthesis of artemisinin. *Phytochemistry*, 68: 1864-1871

Croteau R, Kutchan T, Lewis N (2000) Natural products (secondary metabolites). *In* B Buchanan, W Gruissem, R Joneas, eds, *Biochemistry and Molecular Biology of Plants*. American Society of Plant Biologists, Rockville, MD, pp 1250–1268.

Dalrymple, D.G. (2006) Artemisia, Agriculture and Malaria in Africa: The interplay of Tradition, Science and Public Policy: 2-52

Delabays, N.;Benakis, A.;Collet, G. (1993) Selection and breeding for high artemisinin (*Qinghaosu*) yielding strains of *Artemisia annua*. *Acta Hort. (ISHS)*, 330: 203-208

Dhingra,V.;Vishweshwar, K. R.;Lakshmi M.N.(2000) Current status of artemisinin and its derivatives as antimalarial drugs. *Life Sci*, 66: 279-300

Eckstein-Ludwig, U.; Webb, R.J.; Van Goethem, I.D.; East, J.M.; Lee A.G.; Kimura M., O'Neill, P.M.; Bray, P.G.; Ward SA, Krishna S. (2003) Artemisinins target the SERCA of *Plasmodium falciparum*. *Nature,* 424: 957–961

Ferreira, J. (2004) Artemisia annua L.: The hope against malaria and cancer *Medicinal and Aromatic Plants: Production, Business & Applications*: 56-61

Jiang M.; Zhang, J. (2003) Cross-talk between calcium and reactive oxygen species originated from NADPH oxidase in abscisic acid-induced antioxidant defence in leaves of maize seedlings. *Plant Cell Environ*, 26:929–93

Haynes, R.K.; Krishna, S. (2004) Artemisinins: activates and actions. *Microbes and Infection,* 6: 1339-1346

Hu, X.; Bidney, D.L.; Yalpani, N.; Duvick, J.P.; Crasta, O., Folkerts, O., Lu, G. Overexpression of a gene encoding hydrogen peroxide-generating oxalate oxidase evokes defense responses in sunflower. *Plant Physiol*, 13:170–181

Klayman, D.L. (1989) Weeding out malaria. *Natural History,* 10: 18-91

Klayman, D.L. (1993) *Artemisia annua I*: From weed to respectable antimalarial plant. *In: Kinghorn AD and Balandrin MF (eds), Human medicinal agents from plants*, pp. 242–255. Am Chem Soc Symp Ser Washington D.C.

Krishna, S.; Woodrow, C.J.; Staines, H.M.; Haynes, R.K; Mercereau –Puijalon, O.(2006) Re-evaluation of how artemisinins work in light of emerging evidence of in vitro resistance. *Trends in Molecular Medicine*, 12: 200-205

Kumar, S.; Srivastava, S. (2005) Establishment of artemisinin combination therapy as first line treatment for combating malaria: Artemisia annua cultivation in India needed for providing sustainable supply chain of artemisinin. *Current Science,* 89:1097-1102

Lamb, C.; Dixon, R.A (1997) The oxidative burst in plant disease resistance. Annual Review of Plant Physiology and Plant Molecular Biology, 48: 251–275

Lu, S.; Song, J.; Campbell-Palmer, L. (2008) A modified chemiluminescence method for hydrogen peroxide determination in apple fruit tissues. *Scientia Horticulturae*, 120: 336-344

Mahmoud, S.; Croteau, R. (2001) Metabolic engineering of essential oil yield and composition in mint by altering expression of deoxyxylulose phosphate reductoisomerase and menthofuran synthase. *Proc Natl Acad Sci USA,* 98:8915–8920

Marques,D.A.; Foglio M.A., Morgante, P.G.; Van Sluys, M; Kirszenzaft Shepherd, S.L. (2006) Biotechnology approaches for production of antiulcerogenic dihydro- epideoxyarteannuin B isolated from Artemisia annua L. *Brazilian Journal of Pharmacognosy,* 16: 291-99

Meshnick, S.R.; Taylor, T.E; Kamchonwongpaisan, S. (1996) Artemisinin and the antimalarial endoperoxides: from herbal remedy to targeted chemotherapy. Microbiology and Molecular Biology Reviews, 60: 301-315

Meshnick, S. R. (2002) Artemisinin: mechanism of action, resistance and toxicity. *Int J Parasitol*, 32: 1655–1660

Miller, G.; Suzuki, N.; Rizhsky, L.; Hegie, A.; Koussevitzy, S.; Mittler, R. (2007) Double mutants deficient in cytosolic and thylakoid ascorbate peroxidase reveal a complex mode of interaction between reactive oxygen species, plant development, and response to abiotic stresses. *Plant Physiology*, 144: 1777-1785

Mittler, R.;Vanderauwera, S.; Gollery, M.; Van Breusegem, F. (2004) Reactive oxygen gene network of plants. *Trends Plant Sci*, 9: 490-98

Moller, I.M. (2001) Plant mitochondria and oxidative stress: electron transport, NADPH turnover, and metabolism of reactive species. *Annu Rev Plant Physiol Plant Mol Biol*, 52: 561–591

Noctor, G.; Veljoric-Jovanovic, S.D.; Riscoll, S.; Novitskaya, L.; Foyer, C.H. (2002) Drought and oxidative load in wheat leaves. A predominant role for photorespiration? *Annals of Botany,* 89: 841–850

Olliaro, P.L.; Taylor, W.R. (2004) Developing artemisinin based drug combinations for the treatment of drug resistant falciparum malaria: A review. *Journal of Postgraduate Medicine*, 50: 40-44

Pitzschke, A.;Hirt, H.(2006) Mitogen-activated protein kinases and reactive oxygen species signaling in plants. *Plant Physiol*, 141: 351-56

Rowen, R. J. (2002) Artemisinin: From malaria to cancer treatment http://www.townsendletter.com/Dec2002/artemisinin1202.htm

Shuhua, X.; Tanner, M.; N'Goran, K. E.; Utzinger, J.; Chollet, J.; Bergquist, R.; Minggang, C.; Jiang, Z. (2002) Recent investigations of artemether, a novel agent for the prevention of schistosomiasis japonica, mansoni and haematobia. *Acta Tropica*, 82: 175-181

Stanley Fernandez, S.M.; Kellogg, B.A.; Poulter, C.D (2000) Farnesyl diphosphate synthase. Altering the catalytic site to select for geranyl diphosphate activity. *Biochemistry*, 39: 15316-5321

Wallaart, T.E.; Pras, N.; Quax, W.J. (1999) Isolation and identification of dihydroartemisininic acid hydroperoxide from *Artemisia annua:* A novel Biosynthetic Precursor of artemisinin *J Nat Prod*, 62: 1160-1162

Wallaart, T.E.; Pras, N.; Beekman, A.C.; Quax, W.J. (1999) Seasonal variation of artemisinin and its biosynthetic precursors in plants of *Artemisia annua* of different geographical origin: proof for the existence of chemotypes. *Planta Medica*, 66: 57-62

Weathers, P. J.; Elkholy, S.; Wobbe, K.K. (2006) Artemisinin: The biosynthesis pathway and its regulation in Artemisia annua, a terpenoid-rich species. *In Vitro Cellular & Developmental Biology - Plant*, 42: 309-317

Webster, H. K.; Lehnert E. K. (1994) Chemistry of artemisinin: an overview. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 88: 27-29

WHO (2003) Access to antimalarial medicines: Improving the affordability and financing of artemisinin-based combination therapies http://www.who.int/malaria/cmc_upload/0/000/016/745/37268_ACT_final2.pdf

World Health Organization (2009) Malaria http://www.who.int/mediacentre/factsheets/fs094/en/

Woerdenbag, H.J.; Bos, R.; Salomons, M.C.; et al. (1993) Volatile constituents of *Artemisia annua* L.(Asteraceae) *Flavour and Fragrance Journal*, 8:131-137

van Agtmael M.A.; Eggelte T.A.; van Boxtel C.J. (1999) Artemisinin drugs in the treatment of malaria: from medicinal herb to registered medication. *Trends in Pharmacological sciences*, 20:199-205

Zaninotto, F.; La Camera, S.; Polverari, A.; Delledonne, M.(2006) Cross talk between reactive nitrogen and oxygen species during hypersensitive disease resistance response. *Plant Physiol*, 141: 379-83

Zhang, Y.; Teoh K.H.; Reed, D.W.; Maes, L.; Goossens, A.; Olson D.J.H; Ross, A.R.S.; Covello, P.S.(2008) The molecular cloning of artemisinic aldehyde Δ11 (13) reductase and its role in glandular trichome-dependent biosynthesis of artemisinin in *Artemisia annua. Journal of Biological Chemistry*, 283:21501- 21508

Zhou, B.;Wang, J.; Guo, Z.; Tan, H.; Zhu, X. (2006) A simple colorimetric method for determination of hydrogen peroxide in plant tissues. *Plant Growth Regulation*, 49: 113-18