The Role of Roots in Stimulating Artemisinin Biosynthesis in the Shoots of *Artemisia annua*

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By

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Abstract

Artemisinin, found in *Artemisia annua L.*, is the most effective treatment for malaria and other diseases. Unfortunately, *A. annua* does not produce enough artemisinin to treat the millions of malaria patients; therefore a better understanding of artemisinin biosynthesis is needed. Amorphadiene synthase and CYP71AV1 are the first two enzymes in the pathway, so a better understanding of their expression and regulation is important. In this study, shoots of *A. annua* were inoculated into rooting and shooting media and artemisinin levels and transcript levels of the two enzymes were measured. The results show that roots, or something associated with root development not only stimulated artemisinin production, but also increased the transcript levels of the *ads* and *cyp71av1* genes.

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Table of Content

Table of Figures

Table of Tables

1 Introduction

1.1 Malaria

Currently, malaria is endemic in more than 90 countries in Africa, Asia, South and Central America, and the Eastern Mediterranean Region (Figure 1), where about 40 % of the world population resides (WHO, 2002). Malaria is an infectious disease caused by protozoan parasites. Female *Anopheles* mosquitoes carry these parasites and infect humans while sucking their blood. Among the four types of the parasite, *Plasmodium, P. falciparum* is the most deadly (WHO, 2007a). Everyone is at risk of getting infected, including people traveling from malaria-free countries to malaria infected countries. For example, on April 25, 2008, the World Health Organization (WHO) reported cases of *P. falciparum* found in two tourists visiting the Bahamas, where malaria is not endemic (WHO, 2008a). No one is immune to the parasite therefore they can become infected over and over again. Children and pregnant women are at highest risk of becoming infected (WHO, 2008b). There are more than 500 million infections with a million deaths each year. The parasite has become rather resistant to many drugs, like chloroquine, that were used in the past (Bloland, 2001). According to WHO, unless major steps to control the disease are taken, malaria will increase not only in endemic countries but also in countries where malaria was not prevalent (WHO, 2002).

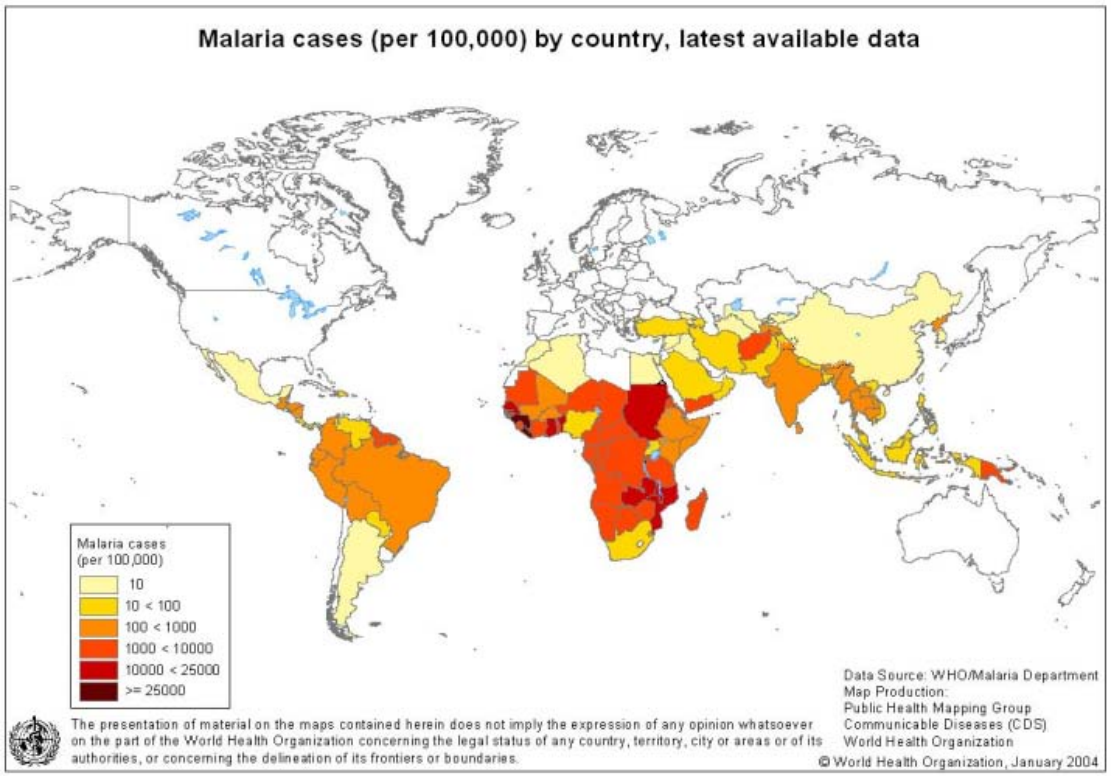
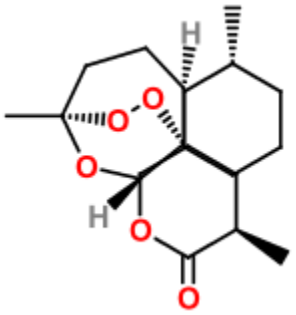


Figure 1: Malaria cases by country. (taken from WHO, 2004)

1.2 Artemisinin, a cure for malaria and other diseases

The chemical, artemisinin (Figure 2), extracted from the plant, *Artemisia annua L.* (Figure 2), cures malaria, especially when used in Artemisinin-based Combination Therapy (ACT) that is aimed at preventing the emergence of drug resistance (WHO, 2006). ACT is a combination of either artemisinin or one of its derivatives, artesunate, artemether, or dihydroartemisinin, with other less effective antimalarial drugs, such as mefloquine or lumefantrine (WHO, 2006). This new treatment has been successful in treating malaria and so far there have been no cases of resistance to the drug. There are few, if any, significant side effect in patients being treated with ACT and artemisinin is safe even for pregnant women (WHO, 2007b).



http://www.alibaba.com/product/bachmann-100001065-0/Artemisia_Annua_Medicinal_Herbs.html

Figure 2: Structure of artemisinin and *Artemisia annua L.*

Not only can artemisinin cure malaria, but it can also be used to treat other diseases, such as sleeping sickness, Chagas' disease, leishmaniasis, schistosomiasis, and different types of cancer (Mashina *et al., 2007;* Avery *et al.*, 2003; Boulanger *et al.*, 2007; Efferth, 2007). Although artemisinin can be used against so many diseases, its limited supply poses a major obstacle. Therefore, a better understanding of its biosynthesis is needed to improve production of this important drug to treat malaria and all the other diseases against which artemisinin is effective.

Since artemisinin and its derivatives have been effective against malaria, there has been an increase in demand for the drug. Unfortunately, this caused a drastic increase in the price because there is a severe shortage of artemisinin (Mutabingwa, 2005). The *A. annua* plant produces, at best, 0.5-1.2% DW artemisinin, so only about 6 to 14 kg of artemisinin can be extracted from 100 acres of plants (Kindermans *et al.*, 2007). For this reason, many studies have focused on other methods to increase the supply of artemisinin while keeping the cost of production to a minimum.

2 Background

It is essential to know more about the pathway leading to artemisinin to further the study of chemical synthesis and metabolic engineering, or to find new ways to increase the production of artemisinin. It is known that two of the enzymes directly leading to artemisinin are amorphadiene synthase (ADS) and a P450, CPY 71AV1. Also, the intact, *A. annua*, plant contains more artemisinin in its shoots than plants without roots (Ferreira and Janick, 1996). Unfortunately, we don't fully understand how these enzymes contribute to the pathway or how they are regulated, where they are expressed, or how the roots affect artemisinin production. The role of the roots on artemisinin production in particular is the focus of this project.

2.1 Role of Roots in Artemisinin Production

Artemisinin is stored only in the trichomes on leaves and flowers (Covello *et al.*, 2007). None is found in the roots, but Ferreira and Janick (1996) found that the artemisinin concentration in shoots with roots, 0.075 % DW (dry weight), is more than its concentration in shoots without roots, 0.035 % DW. This suggested that although the roots do not produce any artemisinin, they somehow play an important role in artemisinin production.

Roots control or affect many shoot responses in plants. An example of root-shoot interaction in plants is the occurrence of hypoxia, which occurs when roots are partly deficient in oxygen (Taiz and Zeiger, 2006b). During hypoxia, the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), is produced in the roots and travels via the xylem to the shoots where it is converted to ethylene by ACC oxidase (Taiz and Zeiger, 2006b). Ethylene-responsive cells in the leaf expand when there is a high concentration

of ethylene and result in epinasty, the downward growth of the leaves so that they appear to droop. Also, in the absence of oxygen, all mechanisms such as oxidative phosphorylation and electron transport in the mitochondria and the tricarboxylic acid (TCA) cycle cease to operate, so ATP can only be produced through fermentation. Since fermentation can only yield 2 moles of ATP per mole of hexose compared to 36 moles of ATP per mole of hexose in aerobic respiration, the plant does not have enough energy to keep itself alive for a long period of time. In another example, under hypoxic conditions older leaves in tomatoes, *Solanum lycopersicum*, show chlorosis and a reduction in growth rate of new leaves (Horchani *et al.*, 2008).

Another root-shoot interaction is the closing of stomates caused by a shortage of water in the soil. Water deficiency in the roots stimulates the accumulation of abscisic acid (ABA) in shoots, then later in roots and guard cells, which signals the closing of stomates. Taiz and Zeiger (2006b) suggest that the lack of an early presence of ABA in the roots resulted in either a rapid transportation of ABA from the roots to the shoots or the roots may transport an ABA precursor in the early stages of water deficiency.

Another example of a mobile signaling factor is the mRNA of *Flowering Locus T (FT)* gene. Although not produced in the roots, the mRNA of the *FT* gene is transported via the phloem from the leaves to the shoot apex and induces flowering in *Arabidopsis* (Huang *et al.*, 2005). The mRNA of the *FT* gene might not be the only mobile flowering stimulus in *Arabidopsis*, however, it does play an important part in flower induction (Huang *et al.*, 2005).

There are many different types of signaling processes in plants so there are a number of signaling factors that could be moving from roots to shoots of *Artemisia annua*

to induce greater artemisinin production in the shoots. This project is designed to determine if the transcription level of the genes for ADS and CPY71AV1 in both the roots and the shoots correlates at all to the presence of roots in the plant.

2.2 Terpenes

There are three main groups of secondary metabolites: terpenes, phenolics, and nitrogen-containing compounds (Croteau *et al.,* 2000). The focus here is on terpenes, the largest group of secondary metabolites that are involved in plant defense against insects and pathogens. Diterpenes and sesquiterpenes, two forms of terpenes, are, for example, produced in rice, *Oryza sativa L.,* in response to fungal infection (Cheng *et al.,* 2007). Commercially, terpenes are used as ingredients in food, in cosmetics products, and as drugs. For example, menthol, a monoterpene (10 carbons), is what gives the mint plant its characteristic scent (Taiz and Zeiger, 2006a), while Taxol ® is a diterpene (20 carbons) anticancer drug produced by the yew tree (Pineiro *et al.,* 2007), and artemisinin is a sesquiterpene lactone (15 carbons) antimalarial drug (Wilkinson and Michlefield, 2007).

2.3 Biosynthesis of IPP and DMAPP

Isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are the isomeric 5 carbon precursors for the synthesis of all terpenes. These two compounds are made in the cytosol using the mevalonate (MVA) pathway and in the plastid using the mevalonate-independent (MEP) pathway (Wilding *et al.,* 2000; Rohdich *et al., 2001*). In the MVA pathway (Figure 3) three acetyl-CoA molecules are catalyzed by thiolase and hydroxymethyglutaryl (HMG)-CoA synthase to produce 3-hydroxy-3-methyglutaryl-

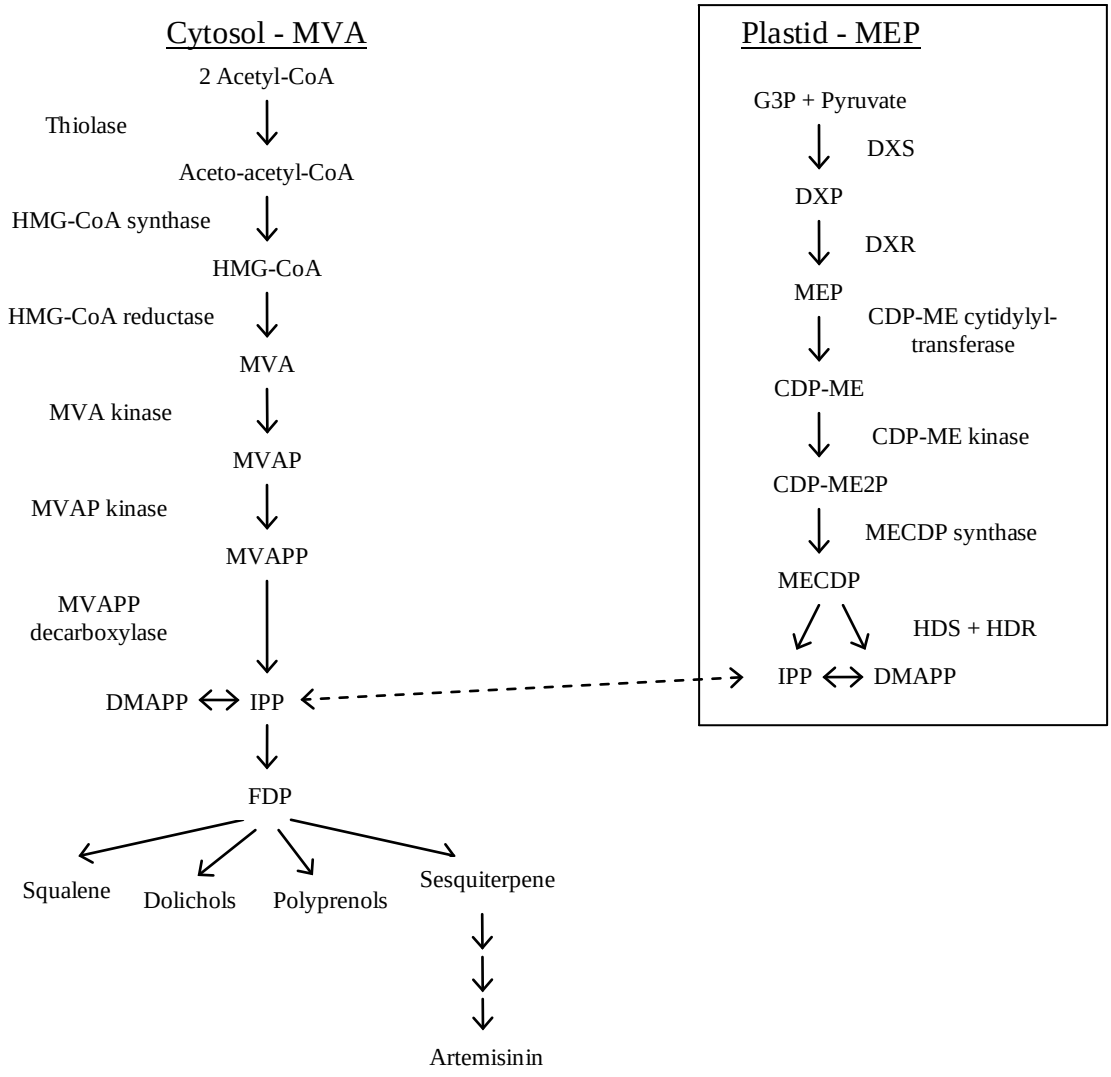


Figure 3: Mevalonate (MVA) and mevalonate independent (MEP) pathways (adapted from Towler and Weathers, 2007). Abbreviations: HMG, hydroxymethyglutaryl; MVA, mevalonate acid; MVAP, mevalonic acid 5-phosphate; MVAPP, mevalonic acid 5-diphosphate; IPP, Isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; FDP, Farnesyl Diphosphate; G3P, glyceraldehyde-3-phosphate; DXS, deoxy-D-xylulose-5-phosphate synthase; DXP, 1-deoxy-D-xylulose-5-phosphate; DXR, 1- deoxy-D-xylulose-5-phosphate reductoisomerase; MEP, methylerythritol phosphate; CDP-ME, 4-diphosphocytidyl-2-C-methyl-D-erythritol; CDP-ME2P, 4-diphosphocytidyl-2-C-methyl-D-erythritol-2-phosphate; MECDP, 2-C-methyl-D-erythritol-2,4-cyclodiphosphate; HDS, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase; HDR, 4-hydroxy-3-methyl-2-(E)-butenyl-4-diphosphate reductase. The dotted line represents an exchange between the cytosol and plastid.

CoA (HMG-CoA) (Croteau *et al.*, 2000). Then HMG-CoA is reduced by HMG-CoA reductase (HMGR) to mevalonic acid (MVA) and MVA is phosphorylated by MVA kinase to mevalonic acid 5-phosphate (MVAP) which is phosphorylated again by MVAP kinase to produce mevalonic acid 5-diphosphate (MVAPP). Finally, MVAPP is decarboxylated by MVAPP decarboxylase to yield isopentenyl diphosphate, or IPP.

In contrast to the MVA pathway, the MEP pathway is localized in the plastid and uses pyruvate and glyceraldehyde-3-phosphate (G3P) as the starting materials instead of acetyl-CoA (Figure 3). Hunter (2007) explained that in the MEP pathway, deoxy-D-xylulose-5-phosphate synthase (DXS) catalyzed the condensation of G3P and pyruvate to form 1-deoxy-D-xylulose-5-phosphate (DXP), which is then reduced by 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) to 2-C-methyl-D-erythritol-4-phosphate (MEP). Next, MEP is converted to 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME) by 4-diphosphocytidyl-2-C-methyl-D-erythritol cytidylyltransferase in the presence of cytidine-5-triphosphate (CTD). Following this, CDP-ME is phosphorylated by CDP-ME kinase to produce 4-diphosphocytidyl-2-C-methyl-D-erythritol-2-phosphate (CDP-ME2P) which is then catalyzed by 2-C-methyl-D-erythritol-2, 4-cyclodiphosphate (MECDP) synthase to form MECDP. Finally, the cyclodiphosphate in MECDP is reduced by two enzymes, 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase (HDS) and 4-hydroxy-3-methyl-2-(E)-butenyl-4-diphosphate reductase (HDR) to form IPP and some DMAPP. Although some DMAPP is formed directly from MECDP, DMAPP is also produced by the isomerization of IPP with isopentenyl-diphosphate isomerase. Both IPP and its isomer, DMAPP, act as the fundamental 5 carbon building blocks of all terpenes.

2.4 Artemisinin Biosynthesis

Until recently, the source of IPP in the biosynthesis of artemisinin was not known. The inhibitors mevinolin (MEV) or fosmisdomycin (FOS) can be used to block HMGR or DXS in the MVA or MEP pathways, respectively. When fed to young *A. annua* seedlings, FOS decreased artemisinin production by about 65%, while in the presence of MEV artemisinin was decreased by about 80% (Towler and Weathers, 2007). In the presence of both inhibitors, MEV and FOS, no artemisinin was produced. This suggested that artemisinin is produced in the cytosol using IPP from both the MVA and MEP pathways.

The artemisinin pathway (Figure 4) begins with the conversion of IPP to farnesyl diphosphate (FPP) by farnesyl diphosphate synthase (FPPase) (Thulasiram *et al.*, 2007). Farnesyl diphosphate is cyclized by amorphadiene synthase (ADS) to form amorpha-4, 11-diene (Kim *et al.*, 2006). Amorpha-4, 11-diene is catalyzed by CYP71AV1 to form artemisinic alcohol then artemisinic aldehyde (Teoh *et al.*, 2006). Artemisinic aldehyde is reduced to dihydroartemisinic aldehyde by the double bond artemisinic aldehyde reductase, Dbr2, (Zhang *et al.*, 2008) and converted to dihydroartemisinic acid (DHAA) by a dehydrogenase (personal communication to P. Weathers from Patrick Covello). Artemisinin is then thought to be produced by a non-enzymatic, spontaneous photo-oxidation of dihydroartemisinic acid (Wallaart *et al.*, 2001).

There are also two other possible pathways to artemisinin via artemisinic acid. They both begin with the conversion of artemisinic aldehyde to artemisinic acid by the enzyme CYP71AV1 (Covello *et al.*, 2007). Next artemisinic acid is catalyzed to form arteannuin B (Brown and Sy, 2007). In one pathway, arteannuin B can be converted directly to artemisinin and in another pathway, arteannuin B can be converted to

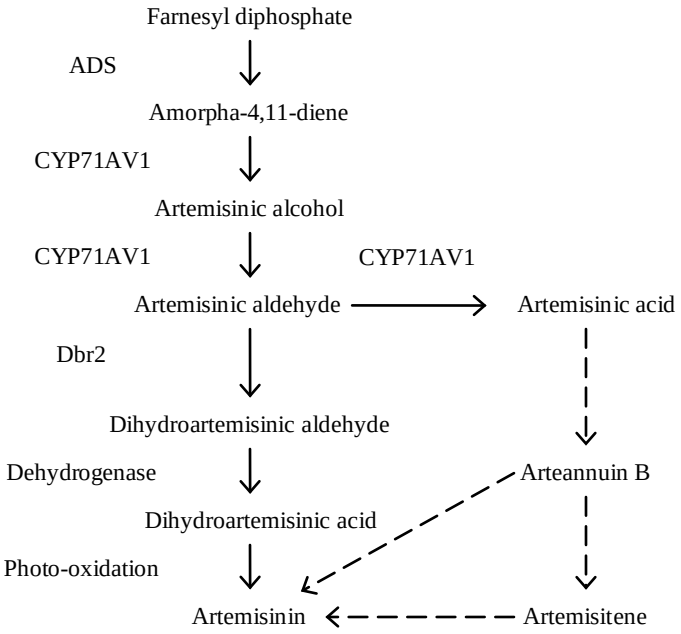


Figure 4: Artemisinin biosynthesis. (adapted from Zhang *et al.*, 2008). Abbreviation: ADS, amorphadiene synthase; CYP, cytochrome P450; Dbr2, artemisinic aldehyde reductase. The dotted lines represent another possible route in the pathway.

artemisitene then to artemisinin (Brown and Sy, 2007). Not much is known about the enzymes and mechanisms involved in these pathways or even if these pathways truly exist *in planta*.

2.5 Regulation of Artemisinin Biosynthesis

Metabolite biosynthesis can be regulated through many mechanisms, such as through genetic or allosteric control of the enzymes involved in the pathway. An example of genetic control is the uses of small interfering RNA (siRNA) and microRNA (miRNA) to control expression of certain proteins. In plants, a dicer-like protein cleaves double stranded RNA and pri-miRNA into 21-23 nucleotides sequences that are double stranded called siRNA and miRNA (Taiz and Zeiger, 2006c). Then an RNA induced silencing

complex (RISC) binds to one strand of the siRNA or miRNA and releases the other strand. Next, RISC begins to search for an mRNA that has a sequence that could base pair with the single stranded RNA bounded to RISC. Once the single stranded RNA binds to the mRNA, RISC will cleave the mRNA and the mRNA gets degraded, which can result in a decrease in synthesis of specific proteins.

Another example of genetic control over protein synthesis is the regulation of transcription. Terpenoid indole alkaloids (TIAs) are secondary metabolites that are known to have many pharmaceutical uses (Memelink *et al*., 2001). For example, the dimeric alkaloid vinblastine is an antitumor drug and the monomeric alkaloid serpentine is use as a tranquillizer. In the TIA biosynthetic pathway, the protein strictosidine synthase is required for the synthesis of strictosidine (Memelink *et al.*, 2001). It was found that in the *Str* promoter region there is a jasmonate- and elicitor-responsive element (JERE) that interacts with octadecanoid-responsive *Catharanthus* AP2-domain proteins (ORCAs). In the presence of jasmonate, *Orca* gene expression is induced and ORCA proteins are synthesized. The ORCA proteins will then bind to the JERE and help activate transcription of the *Str* gene (Memelink *et al.*, 2001).

Another example of regulation is negative feedback inhibition. When reactive oxygen species (ROS) are detected by ROS receptors, redox-sensitive transcription factors and phosphatases, a long process begins that results in production of factors, that regulate gene expression; these include heat shock factors and Myb proteins (Mittler *et al.* 2004). If there are too many heat shock factors or Myb proteins present, they will activate the ROS-scavenging network which in turn inhibits ROS production by the cell.

ROS have been identified as playing a key role in the last photo-oxidation step in artemisinin biosynthesis (Figure 4).

Amorpha-4,11-diene synthase (ADS) and CYP71AV1 (CYP) are important enzymes in artemisinin biosynthesis. ADS is the first enzyme in the pathway and as such could readily be under transcription control. CYP can catalyze up to three of the subsequent reactions (Teoh *et al.,* 2006) and is thus also of interest. Therefore it is essential to study their transcription levels. Since roots seem to be important for increasing artemisinin levels in *A. annua* shoots, there is a possibility that some factor in the roots maybe regulating the transcript levels of the *ads* and *cyp71av1* genes in either the roots or the shoots. This project is designed to determine if the presence of roots have an effect on the transcript levels of either of these genes.

2.6 Production of Artemisinin

Artemisinin has a very complex structure (Figure 2) and although it can be chemically synthesized, it requires many steps and the process is expensive (Yadav *et al.,* 2003). Alternatively, bacteria and yeast have been recently used to also produce the drug. Martin *et al.,* (2003) showed a high level of production of isopentenyl diphosphate (IPP), the 5 carbon precursor of artemisinin, when they engineered the expression of the mevalonate pathway from *Saccharomyces cerevisiae* into *E. coli.* Afterward, they expressed a synthetic amorphadiene synthase gene into the engineered *E. coli* and this resulted in a high yield of amorphadiene (112.2 mg/L after 11 hours). Martin *et al.* (2003) believed that this method could be extended to produce artemisinic acid and then via organic synthesis later yield artemisinin. Research is currently concentrating on

optimizing this method. For example, through the engineered mevalonate pathway, there was an accumulation of HMG-CoA in *E. coli* cells, which severely inhibited growth. It was found that the accumulation of HMG-CoA inhibited enzymes in the fatty acid biosynthesis (FAB) pathway which led to the inhibition of cell growth (Kizer *et al.*, 2008). Other than *E. coli*, yeast (*S. cerevisiae*) have also been used as a host for artemisinin production. Ro *et al.* (2006) engineered a strain of *S. cerevisiae* that increased the production of amorphadiene and decreased the production of sterols, thereby shifting carbon from sterols to sesquiterpenes. Next, Ro *et al.* (2006) added the *A. annua* CYP71AV1 gene to this *S. cerevisiae* strain; the resulting transformed yeast strain produced a high yield of artemisinic acid (4.5% DW in yeast compare to 1.9% DW in *A. annua*)

2.7 Hypothesis

1) If the expression of ADS, CYP71AV1, or both is detected in roots then the roots may help transcribe these genes for artemisinin biosynthesis.
2) If the gene for ADS, CYP71AV1, or both is expressed more in rooted shoots than unrooted shoots, then the roots somehow indirectly affect the transcription of these genes in shoots.

2.8 Objectives

1) To measure the artemisinin level of rooted shoots and unrooted shoots every four days for 24 days.
2) To analyze the transcript level of *ads* and *cyp71av1* gene in rooted shoots and unrooted shoots
3) To compare the artemisinin levels with the transcript levels of these genes in rooted shoots and unrooted shoots.

3 Methodology

3.1 Cultures, Root Induction, and Shoot Maintenance

Shoot cultures of the Chinese strain (CH) of *Artemisia annua* (gift of C.Z. Liu) were grown in Magenta boxes containing 50 ml of shooting medium containing 4.44 g/L MS salts (Phytotechnology Laboratories; Prod. # M404; Murashige and Skoog, 1962), 0.25 µM NAA (1-naphthaleneacetic acid; Sigma, cat. # N0640-256), 2.5 µM BAP (6-benzylaminopurine; Sigma, cat. #B3408-5G) and 3% (w/v) sucrose under constant cool-white fluorescent light (20-30 µmol m^{-2} s^{-1}) for about 24 days. One gram fresh weight of shoot tissue was inoculated into a 250 ml Erlenmeyer flask containing 10 ml of sterile liquid shooting medium (see above) or rooting medium containing ½ MS (2.22 g/L) and 2% (w/v) sucrose. The flasks were placed on a shaker at 95 RPM under constant cool-white fluorescent light (20-30 µmol m^{-2} s^{-1}). Every four days for 24 days, the medium was removed and replaced with fresh medium: the old medium was discarded, and then the plants were rinsed with sterile water while still in the flasks. After the third rinse, 10 ml of fresh rooting medium containing ½ MS (2.22 g/L) and 2% sucrose was deposited inside each flask. Every four days, three flasks were harvested just before fresh media was exchanged. The experiment described above was run again for 10 days except this time there was no media exchanged on subsequent days. Cultures were harvested on day 8 and day 10; at day 10, cultures had essentially run out of medium. Shoots were dried, extracted, and assayed for artemisinin using HPLC.

Shoot cultures that were grown in the same way described above were used in another experiment. Shoot were excised from ~20 day old stock cultures growing in shooting medium in Magenta boxes and inoculated into another Magenta box containing

fresh shooting or rooting medium. Every eight days for 24 days, five cultures were harvested, shoots were dried and extracted, and analyzed for artemisinin using HPLC.

To test the effect of shooting medium on rooted cultures and production of artemisinin, seven day old rooting cultures grown in semi-solid rooting medium were transferred into Magenta boxes containing semi-solid shooting or rooting medium. After three days, five cultures were harvested, shoots were dried, and artemisinin was extracted and analyzed using HPLC.

3.2 RNA Extraction of Shoots and Roots

Harvested cultures were drained of medium, rinsed thrice with dH_20, and the shoots and roots from each flask were separated and individually frozen in liquid nitrogen. While frozen, the tissues were ground into a fine powder with a cold mortar and pestle. The ground tissue samples were then stored at -80 °C until RNA extraction. For RNA isolation, 50-100 mg of the frozen ground tissue was mixed with 0.5 ml of PureLink Plant Reagent (Invitrogen, Carlsbad, CA, cat. # 12322-012). The solution was incubated for 5 minutes at room temperature, and then centrifuged at 12,000 x g for 2 minutes. The supernatant was transferred to a fresh 1.5 ml Eppendorf tube and mixed with 0.1 ml of 5 M NaCl and 0.3 ml of chloroform. The solution was thoroughly mixed and centrifuged at maximum speed for 10 minutes at +4 °C. The upper, aqueous phase was placed in a new 1.5 ml Eppendorf tube and 0.4 ml of isopropyl alcohol was added. After incubating for 10 minutes on ice, it was centrifuged at maximum speed for 10 minutes at room temperature. One ml of 75 % ethanol was added to the pellet and the mixture was centrifuged at 12,000 x g for one minute at room temperature. The pellet was resuspended in 20 µl of RNase-free water and stored at -80 °C.

The Turbo DNA-free kit (Ambion, Austin, TX, cat. # AM1907) was used to remove any genomic DNA left in the RNA extraction according to the manufacturer's specifications: a maximum of 10 ug of nucleic acid was added to 50 µl DNase reaction, containing 1X DNase buffer, DEPC-treated water, and 4 U DNase. The solution was incubated for 30 minutes at 37 °C, and then another 4 U of DNase was added. Finally, it was incubated for another 30 minutes at 37 °C.

3.3 Reverse-Transcription and PCR

The DyNAmo cDNA synthesis kit (New England Biolabs, Ipswich, MA, cat. # F-470L) was used to reverse-transcribe RNA transcripts to cDNA according to the manufacturer's specifications. To reverse-transcribe both mRNA and the 18S rRNA, used for an internal reference, random hexamers were used for cDNA synthesis instead of oligo-dT primers. The reverse transcription reaction was incubated for 1 hour at 37 °C, and aliquots were added directly to PCR reactions.

The reagents used for real-time PCR in the Bio-Rad iCycler Real-time PCR system were part of the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, cat. #170-888). In the procedure used, there was a three-step amplification followed by a melt-curve analysis; this was needed because a melting curve shows how pure DNA is in a reaction. For each amplification cycle, there was a denaturation step at 94 °C, an annealing step at 53 °C, and an extension step at 72 °C. A total of 35 cycles was used. Relative fold changes in gene expression were calculated based on the $2^{-\Delta\Delta CT}$ comparative method (Livak and Schmittgen, 2000; Sehringer et al., 2005; Cikos et al., 2007). First, the levels of ADS and CYP71AV1 amplification in experiment samples were normalized to the levels of a normalizing gene, the 18S ribosomal small subunit. Then, the new levels

of ADS and CYP71AV1 amplification of one experimental sample were compared to another sample or to the control. The 18S ribosomal gene was used because it has high expression, it is stable, and, most importantly, it is transcribed constitutively in all conditions and tissues (Deprez et al., 2002; Thellin et al., 1999; Schmittgen and Zakrajsek, 2000; Brunner et al., 2004). The primers for the amplification of the four genes were obtained from Vail (2008) and are listed in Table 1.

Gene	Direction	Sequence (5' => 3')	Base Pairs	Product Length
ADS	Forward	ATACAACGGGCACTAAAGCAACC	23	297 bp
ADS	Reverse	GAAAACTCTAGCCCGGGAATACTG	24	297 bp
CYP	Forward	GGGGTTAGGGATTTAGCCAGAA	22	218 bp
CYP	Reverse	AATTGCCTCCAGTACTCACCATAA	24	218 bp
HMGR	Forward	GGTCAGGATCCGGCCCAAAACATT	24	251 bp
HMGR	Reverse	CCAGCCAACACCGAACCAGCAACT	24	251 bp
18S	Forward	TCCGCCGGCACCTTATGAGAAATC	24	219 bp
18S	Reverse	CTAAGAACGGCCATGCACCACCAC	24	219 bp

Table1. Primer sequences for target gene amplification by RT-PCR.
Abbreviations: ADS, amorpha-4,11-diene synthase; CYP, P450 CYP71AV1;HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase;18S, 18S ribosomal small subunit.

3.4 Artemisinin Extraction and Analysis

After the dry weight of harvested *A. annua* shoots was obtained, they were ground and 2 ml of toluene was added to each sample in a test tube. The samples were then sonicated for 30 minutes in a cold water bath then the extract was decanted into a new set of test tubes. The tissues were extracted twice again with 2 ml of toluene each time. The

three liquid extracts of each sample were pooled and dried under nitrogen gas with an N-evaporator at 30 ºC. The samples were stored at -20 ºC until HPLC analysis.

Dried extracts were analyzed by HPLC using the Q260 assay for artemisinin described by Towler and Weathers (2007). The dried extracts were resuspended in100 µL methanol and then 400 µL of 0.2% NaOH were added. The samples were capped, vortexed, and heated for 35 minutes at 50 ºC in a heating block. Next, the sample tubes were placed in a beaker of ice water and 400 µL of 0.2 acetic acid and 100 µL methanol were added. After vortexing again, the samples were filtered through 0.46 µm filter syringe into a clean HPLC injector vial, capped, and placed in the HPLC auto sampler for analysis. HPLC separation occured on a C18 column (15 cm x 4.6 mm, 5 µm particle size) with an isocratic mobile phase of 40% (v/v) methanol and 60% 0.01 M sodium phosphate at pH 7.0. Detection was at 260 nm. Artemisinin was identified by elution time equal to that of an authentic standard.

3.5 Statistical Analysis

All experiments consisted of at least three replicates and all values were expressed as the mean ± standard deviation. Statistical differences were determined by averaging and comparing the results against the controls. Statistical analyses were performed using the T-test.

4 Results and Discussion

In order to determine the relationship between the roots of *A. annua*, production of artemisinin, and the transcription of the enzymes ADS and CYP71AV1, mRNA of shoots and roots of *in vitro* plantlets was extracted and real time PCR was done to measure the level of mRNA transcripts of these two enzymes. From the harvested shoots, RNA was extracted every four days after shoots were transferred into rooting medium. Biomass of shoots and roots and artemisinin levels were also measured in these rooted shoots every four days.

4.1 Biomass and Artemisinin Concentration of Rooting Plantlets

Shoot cultures of *A. annua* were grown in rooting medium for 20 days. Every four days, three flasks were harvested and tissues dried and then the medium was decanted and replaced with 10 ml of fresh medium. Figure 5 shows the dry weight of the shoots (blue line) and roots (red line) of the harvested plants grown in liquid rooting medium along with the dry weight of shoots (purple line) that were grown in liquid shooting medium that were harvested every 8 days. The shoot cultures grown in shooting medium continued to grow over a period of 15 days and the total biomass of these shoots on day 8 is almost twice as much as the biomass of the shoots grown in rooting medium, 0.23 g to 0.14 g, respectively. The shoots of the cultures grown in rooting medium also increased from 0.05 g at day 0 to about 0.17 g at day 12. The cultures grown in rooting medium began to root on day 8 and root biomass increased rapidly after day 12. Total biomass of cultures grown in shooting medium was always greater than cultures grown in rooting medium.

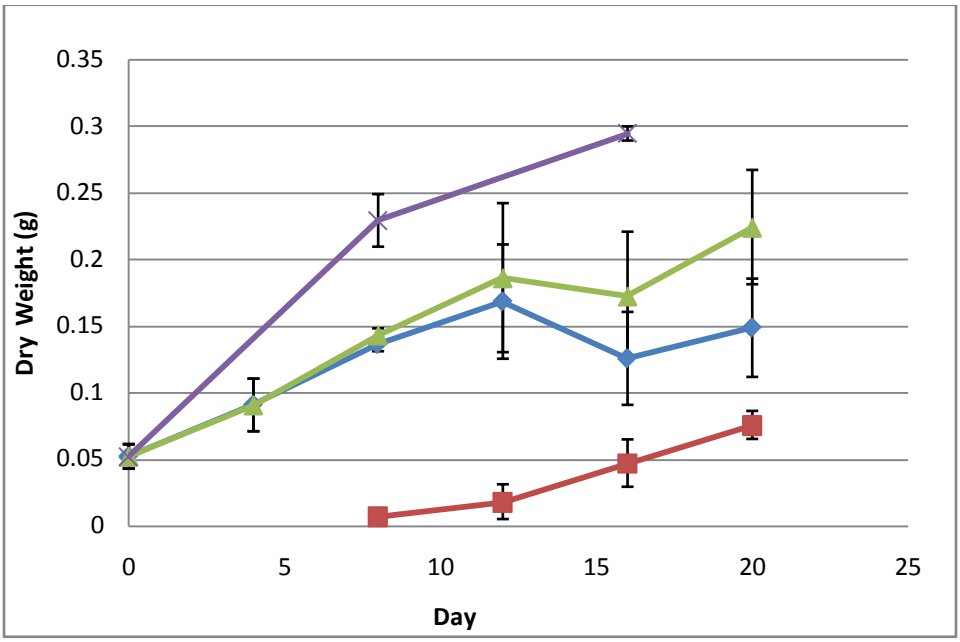


Figure 5: Growth of *A. annua* roots and shoots in liquid MS medium that was refreshed every four days. Control was grown in shooting medium and experimental was grown in rooting medium.

Key: shoot mass of rooted shoots; root mass of rooted shoots; total biomass of rooted shoots; shoot mass of unrooted shoots

The artemisinin level of the shoots grown in liquid rooting medium was measured, but the data were not significantly different than shoots growing in shooting medium (Figure 6A). Abdul Manan, a visiting graduate student at Arkansas State University, also did the same experiment. His data are shown in Figure 6B. Similar to Figure 5, his results show that the growth of *A. annua* (SH DW) shoots grown in rooting medium increased over about 8 days, The roots in these cultures (RT DW) began to form on day 8 and then at day 12 rapidly increased in dry mass. However, contrary to Ferreira's and Janick's (1996) observation, artemisinin level of the rooted cultures (SH AN) did not increase significantly in the shoots as the roots began to form.

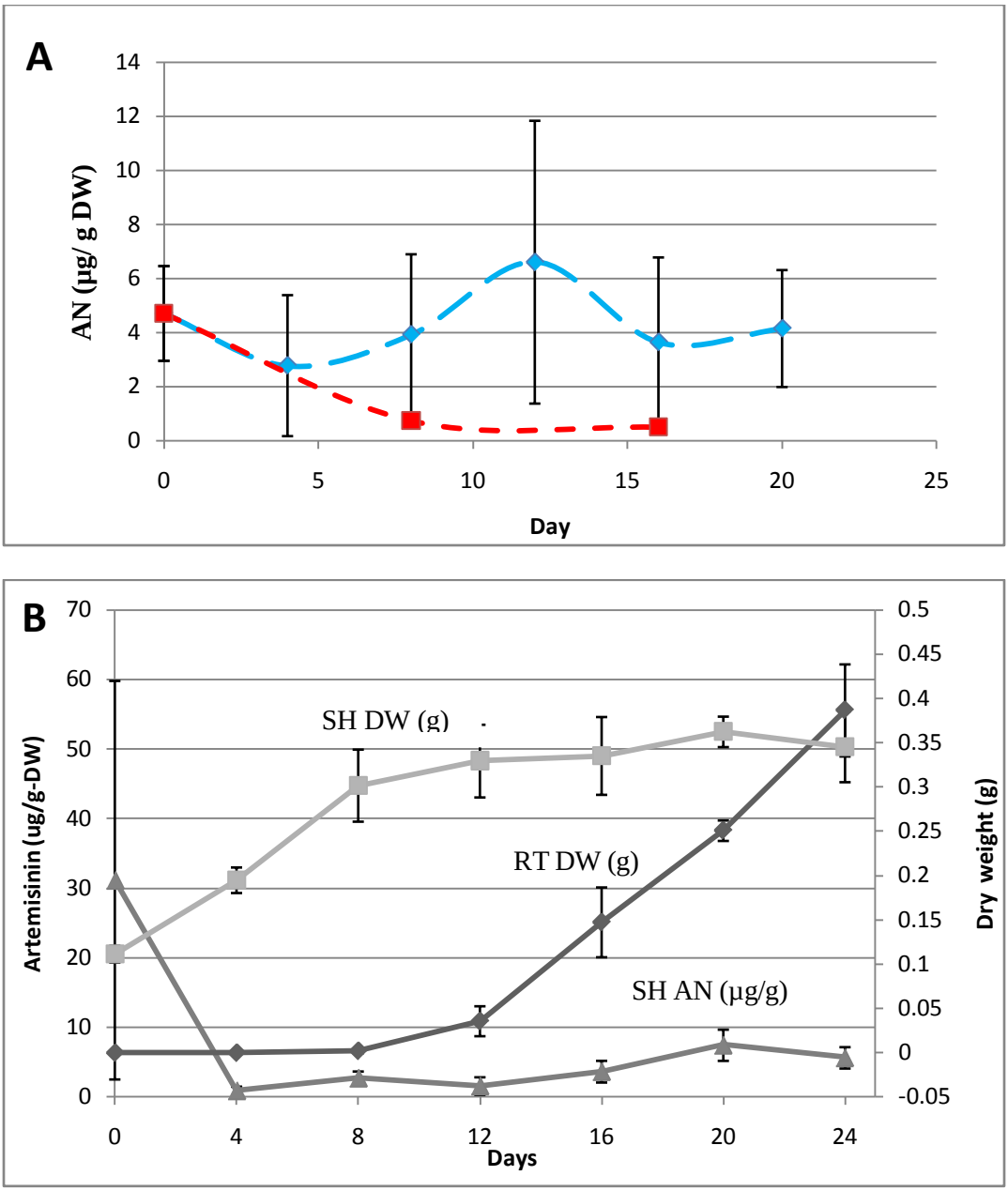


Figure 6: Biomass and artemisinin level of *A. annua* cultures grown in liquid medium that was refreshed every four days over a period of 24 days

Key: artemisinin level of rooted shoots; artemisinin level of unrooted shoots

Abbreviations: SH DW, dry weight of shoots; RT DW, dry weight of roots; SH AN, artemisinin level of shoots

Ferreira and Janick (1996) stated that intact plants produce more artemisinin than plants without roots so it was expected that rooted shoots should consistently produce more artemisinin than unrooted shoots. However, as shown in Figure 6B, at inoculation (day 0) artemisinin level was 6 fold greater than even the oldest rooted shoots (day 20-24). A possible explanation for this unexpected result is that in both experiments shown in Figure 6 A and B, old media was replaced with fresh media every four days. It is possible that periodic refreshing of the medium interferes with artemisinin production. To test this hypothesis, unrooted shoots were inoculated into Magenta boxes and grown in the same medium for 24 days. Figure 7 compares the artemisinin level of rooted and unrooted cultures in solid rooting and shooting media, respectively. Similar to the results reported by Ferreira and Janick (1996), the rooted cultures produced significantly more artemisinin than unrooted cultures after day 8. Interestingly, the unrooted cultures also continued to produce more artemisinin over time, but at a much lower level. The 24 day old cultures in shooting medium (Figure 7) represent the age of cultures used at inoculation for the experiments previously done using shake flask (Figure 6 A and B). The initial artemisinin level at inoculation was greater than any of the shoots of the rooted cultures even after 24 days growth (Figure 6B). These results indicate that shoots at inoculation grown in shooting medium for 20-24 days have an increasing amount of artemisinin, thereby explaining the high level of artemisinin at inoculation for results shown in Figure 6 A and B. Together these data suggested that the change in medium not only disrupted the artemisinin production, it also decreased artemisinin levels right after the medium change. Results, however, needed to be similarly demonstrated in liquid medium.

Figure 8 shows the results for shoots grown in liquid rooting medium for 10 days where medium was not replenished every four days. This experiment was done over a period of 10 days instead of 20, like the ones from Figure 6, because most of the medium was depleted by day 10. Similar to Figure 7, these results show that artemisinin levels increased, from 49.2 µg/ g DW at day 8 to 130.1 µg/ g DW at day 10, as roots began to form. It also further supports the conclusion that the data shown in Figure 6 likely resulted from the frequent change in media. When the media were not replenished, the artemisinin level on day 8 of rooted shoots was 49.2 µg/ g DW and when the medium was frequently refreshed, the artemisinin level on day 8 of rooted shoots was 3.9 µg/ g DW.

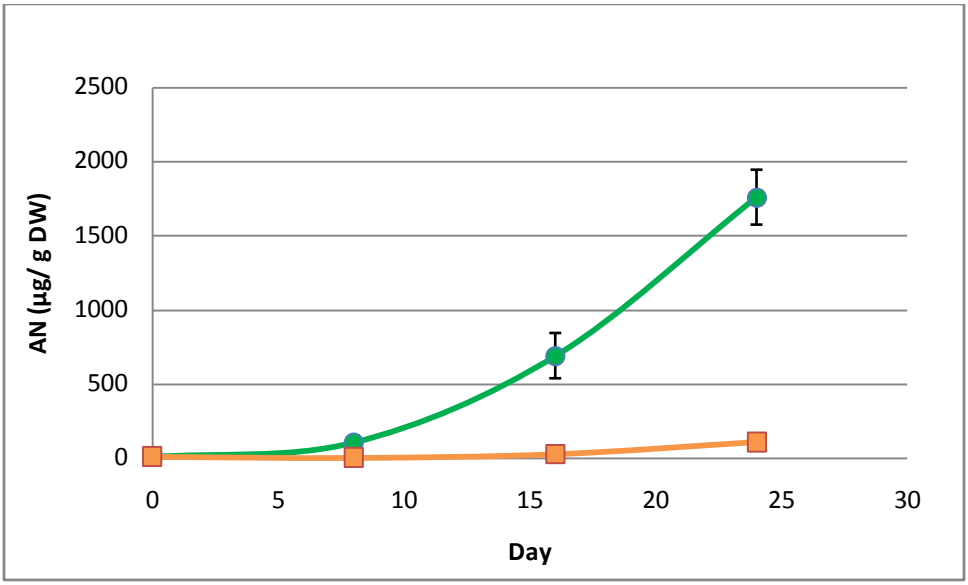


Figure 7: Artemisinin level of *A. annua* cultures grown in Magenta boxes over a period of 24 days.

Key: artemisinin level of shoots in rooting medium; artemisinin level of shoots in shooting medium

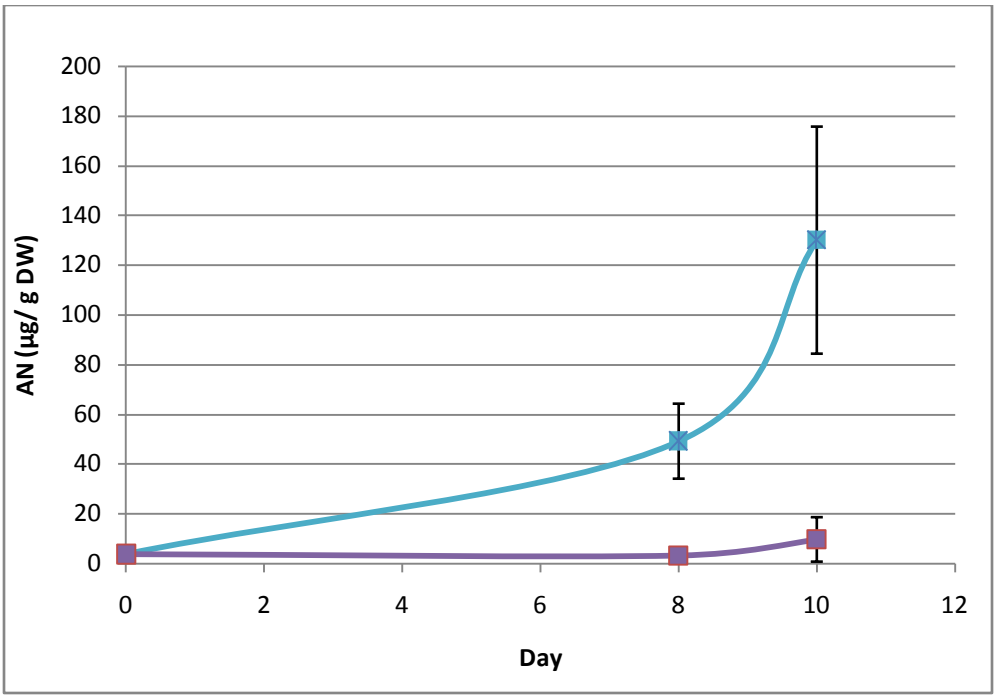


Figure 8: Artemsinin level of *A. annua* cultures grown in liquid medium that was not refreshed over a period of 10 days

Key: artemisinin level of shoots in rooting medium; artemisinin level of shoots in shooting medium

An alternative explanation of the results in Figure 8 may be that the media composition is affecting the artemisinin level. Shooting medium contains two potent phytohormones, NAA and BAP, while rooting medium has none. It is, thus, possible that these two hormones are inhibiting artemisinin production to some degree. To test this hypothesis, intact plantlets grown in solid rooting medium were transferred into both solid rooting and solid shooting media in Magenta boxes and then harvested on three days later. Figure 9 shows the artemisinin level of the shoots grown in rooting media and shooting media. The artemisinin levels of shoots in rooting and shooting medium were not statistically different, suggesting that compared to rooting medium the presence of the phytohormones, NAA and BAP, may not have a major effect on artemisinin production.

This experiment was done using semi solid medium and roots could have been damaged during transfer to new medium. Repeating this experiment in liquid medium and maintaining the cultures for more than three days post transfer could verify results from this preliminary experiment.

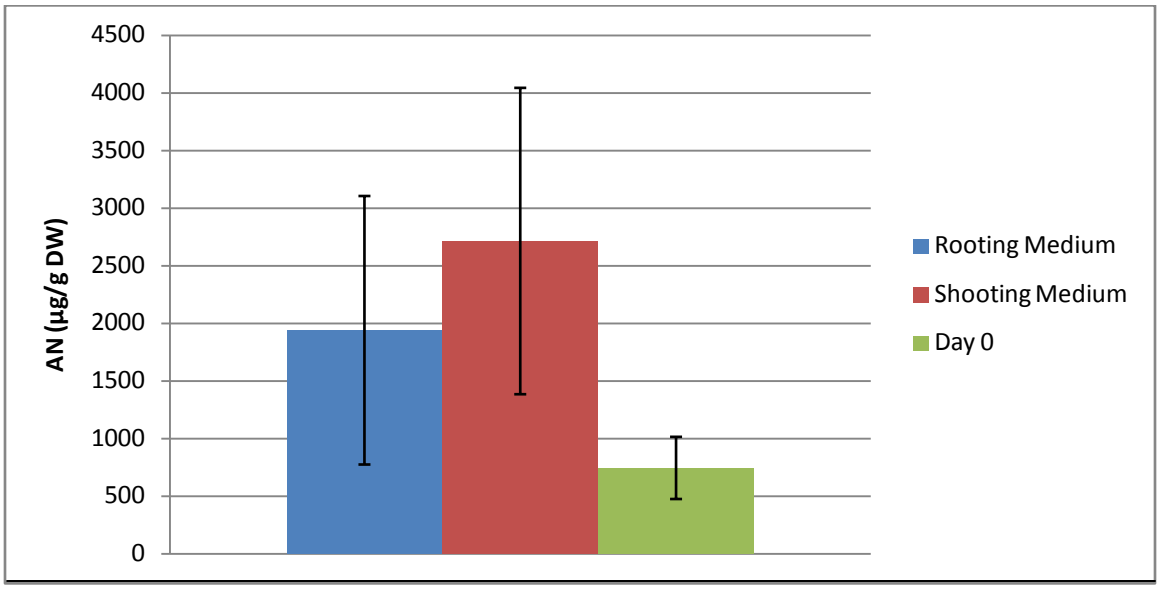


Figure 9: Artemsinin level of *A. annua* cultures grown in solid rooting medium and shooting medium over a period of 3 days

4.2 Transcription Level of mRNA of Plantlets

To determine how transcription of ADS and CYP was affected throughout the rooting process, RNA was extracted and the level of the ADS and CYP71AV1 transcripts were measured in both the roots and the shoots of cultures provided with fresh medium every 4 days for 20 days. Figure 10 shows the level of mRNA transcripts of the *ads* and *cyp71av1* genes measured at days 8 and 16 in cultures grown in rooting medium relative to day 0 cultures (at inoculation). At day 8 and day 16 the transcript levels of the *ads*

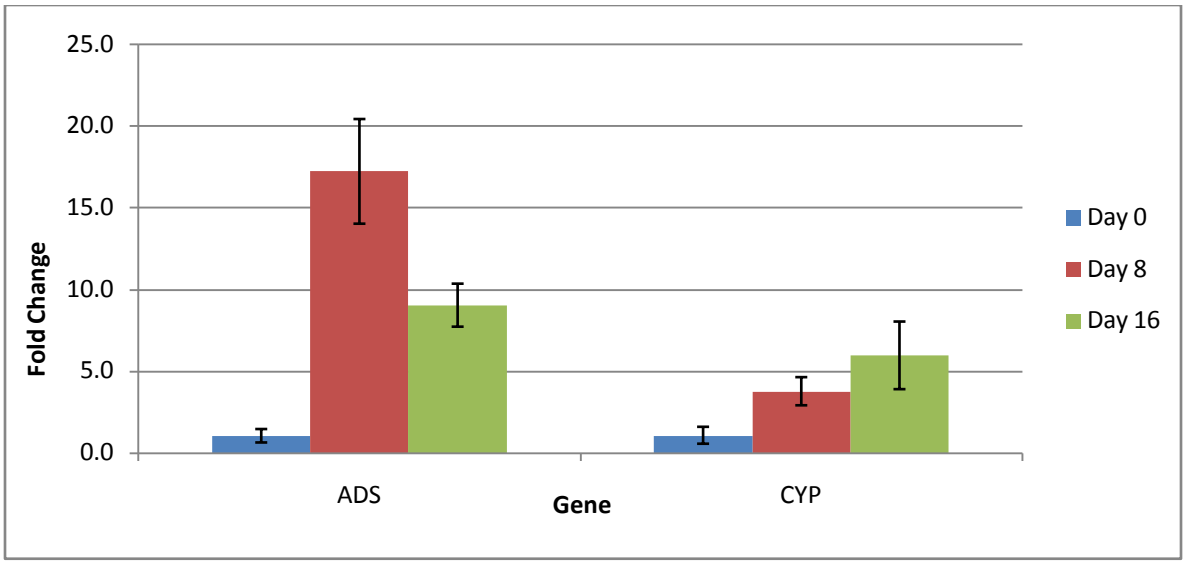


Figure 10: Transcription level of mRNA of ADS and CYP in *A. annua* shoots grown in liquid medium for day 8 and day 16 relative to cultures at day 0 (at inoculation).

and *cyp71av1* genes are more than 9 and 3 fold higher, respectively, than the transcription level of these genes at day 0. The transcript level of *ads* increased significantly from day 0 to day 8, but then decreased from day 8 to day 16. As for the *cyp71av1* gene, transcript levels slowly increased to about 6 fold from day 0 to day 16.

It is more important, however, to compare the transcript levels of these two genes in rooted cultures with expression in unrooted cultures of the same age. Figure 11 A and B show the transcript levels of *ads* and *cyp71av1* in shoots of the cultures grown in rooting medium compared to the cultures grown in shooting medium. On day 8, when roots are first visible, the transcript levels of *ads* in rooted shoots are twice that of unrooted shoots. Transcripts of *cyp71av1*, however, are not significantly different. In contrast, at day 16, transcript levels of both *ads* and *cyp71av1* increased eight and six fold, respectively, for cultures grown in rooting media compared to cultures grown in shooting media.

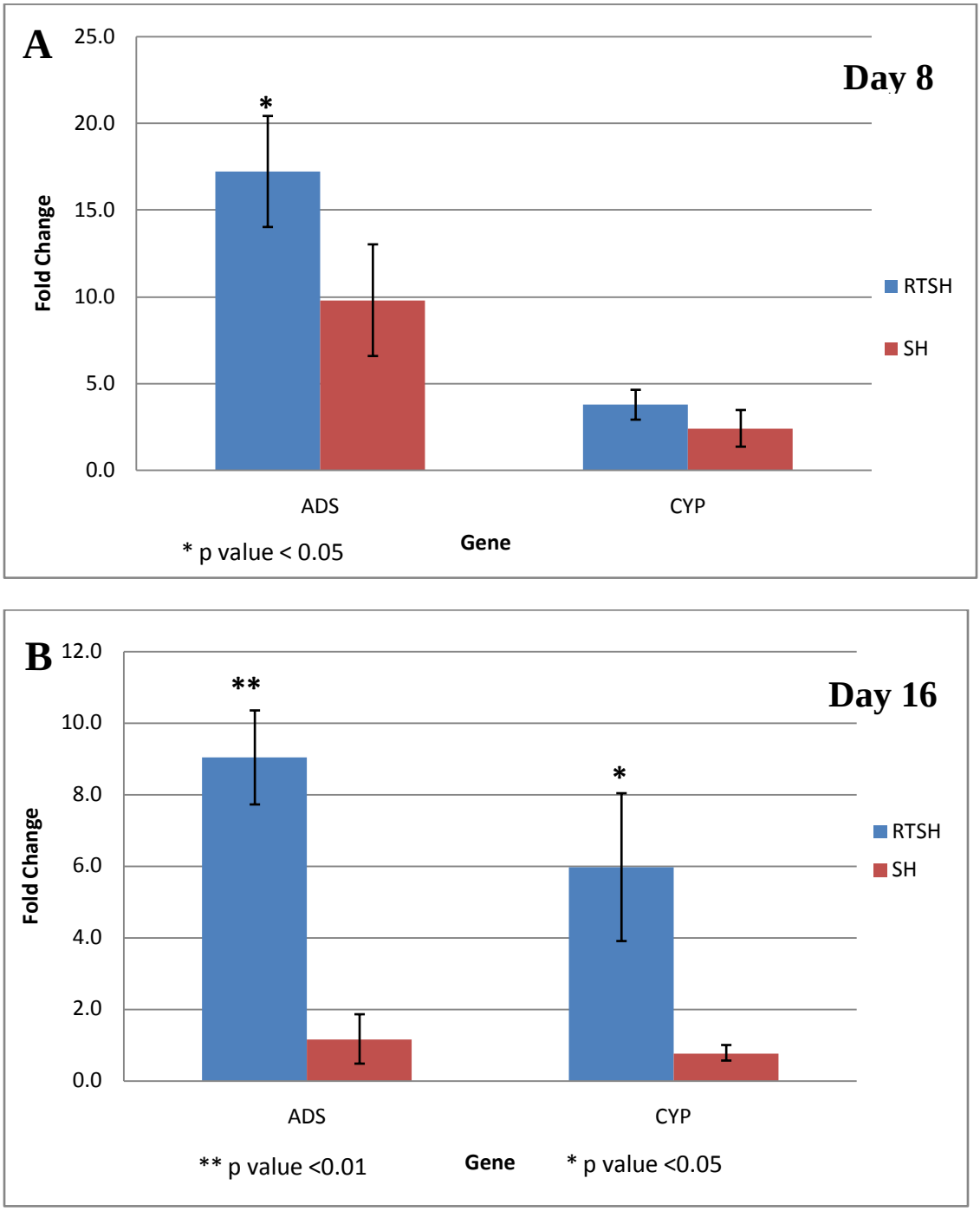


Figure 11: Transcription level of mRNA of ADS and CYP in *A. annua* shoots grown in liquid rooting medium relative to cultures grown in shooting medium on day 8 (A) and day 16 (B).
Abbreviations: RTSH, transcript level of rooted shoot; SH, transcript level of unrooted shoot

When the mRNA transcript levels of both of these genes in rooted shoots are compared to the unrooted shoots, the levels in the rooted shoots are always higher. This indicates that formation of roots affects artemisinin biosynthesis by directly or indirectly increasing RNA transcripts of *ads* and *cyp71av1* in the shoots. Although both genes exist in roots (Teoh *et al.*,2006; Weathers *et al.*, 2006), transcript levels of *ads* and *cyp71av1* in roots of plantlets grown in rooting medium were not detectable in this study.

5 Conclusions and Future Work

Since there is a possibility that the difference between the composition of rooting and shooting media is affecting the artemisinin biosynthesis pathway, we cannot conclude that roots stimulate artemisinin production or increase *ads* and *cyp71av1* transcript levels. It can be seen from the presented data that either something in the roots is stimulating artemisinin production and increasing *ads* and *cyp71av1* transcription or that whatever it is that is stimulating artemisinin production and increasing *ads* and *cyp71av1* transcription is also stimulating root formation. The correlation between root formation and artemisinin production is still unclear; therefore more studies are needed before any conclusion can be made.

The results in this report show that artemisinin levels of cultures grown in Magenta boxes are much higher than those grown in flasks. For example, on day 8, the artemisinin level of rooted shoots grown in Magenta boxes is 110 µg/ g DW, while that of rooted shoots grown in flasks without replenishing the media every four days is only 49.2 µg/ g DW. Magenta boxes and flasks are very different from each other. Magenta boxes are more closely sealed than flasks therefore there is little gas exchange, and differences in the headspace gas may be affecting artemisinin production. To better understand this phenomenon, cultures should be grown in liquid in tightly sealed flasks to see if there is a significant increase in artemisinin level compare to flasks that are not tightly sealed.

Another study that could be done is a repeat of the experiment in Figure 9 where rooted shoots grown in rooting medium are inoculated into shooting medium to see if the difference in medium composition affects artemisinin production. Unlike the preliminary experiment, this one should be done in liquid medium so that there is little damage to the

roots during transfer of the cultures into the alternate medium. For both of these experiments, transcript levels of *ads* and *cyp71av1* should also be measured to see if there is a correlation between the stimulation of artemisinin production and *ads* and *cyp71av1* expression.

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